University of Alberta

Interspecific cross of *Brassica napus* x *B. oleracea* var. *alboglabra*: the effect of growth condition and silique age on hybrid production, and the inheritance of erucic acid in the self-pollinated backcross generation

by

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Abstract

The objectives of this study were to (i) investigate the effects of silique age (as measured by days after pollination) and growth condition (temperature) on the efficiency of ovule culture embryo rescue technique for production of interspecific hybrids from reciprocal crosses between *Brassica napus* (2n=38, AACC) and *B. oleracea* var. *alboglabra* (2n=18, CC), and (ii) to study the inheritance of erucic acid in self-pollinated BC_1 plants of (*B. napus* x *B. oleracea* var. *alboglabra*) x *B. oleracea* var. *alboglabra* i.e. in BC_1S_1 seeds. The greatest numbers of hybrid embryos per pollination were produced under 20°/15°C (day/night) at 16 DAP for *B. oleracea* x *B. napus* crosses, while under 15°/10°C at 14 DAP for *B. napus* x *B. oleracea* crosses. Gametes of F_1 and BC_1 plants containing a greater number of A-genome chromosomes appeared to be more viable, as evidenced from the inheritance of erucic acid in BC_1S_1 seeds and occurrence of a high proportion of intermediate and *B. napus*-type plants in the BC_1 generation.

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Table of Contents

Chapter 1: Literature review

1.1 INTRODUCTION 1
1.2 GENETIC DIVERSITY IN *B. NAPUS* 3
1.3 CANOLA MEAL AND OIL QUALITY 7
1.3.1 GENETICS OF ERUCIC ACID 10
1.3.2 GENETICS OF SEED GLUCOSINOLATE 12
1.4 WIDE HYBRIDIZATION IN *BRASSICA* 14
1.4.1 *BRASSICA* GENOME RELATIONSHIPS 15
1.4.2 HYBRIDIZATION BARRIERS 17
1.4.3 EMBRYO RESCUE TECHNIQUES 18
1.5 RESEARCH OBJECTIVES 20
1.6 REFERENCES 21

Chapter 2: Effects of temperature and age of siliques on hybrid embryo yield from interspecific crosses between *Brassica napus* and *B. oleracea* var. *alboglabra*

2.1 INTRODUCTION 30
2.2 MATERIALS AND METHODS 32
2.2.1 PARENT MATERIAL 32
2.2.2 EXPERIMENTAL DESIGN 32
2.2.3 OVULE CULTURE 33
2.2.4 CONFIRMATION OF HYBRIDS USING SSR MARKERS 34
2.2.5 STATISTICAL ANALYSIS 35
2.3 RESULTS 36
2.3.1 F_1 HYBRID 36
2.3.1.1 SILIQUE SET AND DEVELOPMENT 36
2.3.1.2 FERTILIZED OVULES 37
2.3.1.3 F_1 HYBRID EMBRYO PRODUCTION 39
2.3.1.4 CONFIRMATION OF THE F_1 HYBRIDS 41

2.3.1.5 F_1 PLANT MORPHOLOGY 42
2.3.2 BACKCROSSES 45
2.4 DISCUSSION 47
2.5 REFERENCES 49

Chapter 3: The inheritance of erucic acid in a self-pollinated backcross generation (BC_1S_1) of interspecific crosses between *Brassica napus* and *B. oleracea* var. *alboglabra*

3.1 INTRODUCTION 53
3.2 MATERIALS AND METHODS 55
3.2.1 PARENT MATERIAL 55
3.2.2 EXPERIMENTAL PROCEDURE 56
3.2.3 BC_1S_1 SEED SET: MANUAL VS. BAG ISOLATION 56
3.2.4 POLLEN FERTILITY 56
3.2.5 FATTY ACID ANALYSIS 57
3.2.6 ERUCIC ACID SEGREGATION 58
3.2.7 STATISTICAL ANALYSIS 61
3.3 RESULTS 62
3.3.1 BC_1 MORPHOLOGY 62
3.3.2 BC_1S_1 ERUCIC ACID INHERITANCE 63
3.3.2.1 FLOWER COLOUR AND ERUCIC ACID 66
3.4 DISCUSSION 66
3.5 REFERENCES 69

Chapter 4: Conclusions

4.1 CONCLUSIONS 71

APPENDIX A 73

List of Tables

TABLE 1-1. OILSEED PRODUCTION (IN 1,000 TONNES) IN SELECTED COUNTRIES FOR 2004 (FAO STATISTICAL YEARBOOK 2005) 2

TABLE 1-2. SELECTED ERUCIC ACID ALLELES DESCRIBED IN THE LITERATURE; REVIEWED BY GETINET ET AL. (1997). GENE NOMENCLATURE WAS PROPOSED BY KRZYMANSKI AND DOWNEY (1969) 11

TABLE 1-3. EMBRYO RESCUE TECHNIQUES APPLIED FOR INTERSPECIFIC CROSSES BETWEEN *B. NAPUS* AND *B. OLERACEA* 20

TABLE 2-1. NUMBER OF FERTILIZED OVULES FOR SILIQUES HARVESTED FROM 6 TO 16 DAP FOLLOWING RECIPROCAL INTERSPECIFIC CROSSES BETWEEN *BRASSICA NAPUS* AND *B. OLERACEA* VAR. *ALBOGLABRA* 38

TABLE 2-2. TOTAL NUMBER OF HYBRID EMBRYOS FROM SILIQUES HARVESTED DURING THE WHOLE PERIOD OF 6 TO 16 DAP AND DEVELOPED UNDER TWO TEMPERATURE CONDITIONS FOR RECIPROCAL CROSSES BETWEEN *B. NAPUS* AND *B. OLERACEA* VAR. *ALBOGLABRA* 40

TABLE 2-3. *IN VIVO* INTERSPECIFIC HYBRID PRODUCTION FOR RECIPROCAL CROSSES BETWEEN *B. NAPUS* X *B. OLERACEA* VAR. *ALBOGLABRA* 41

TABLE 2-4. NUMBER OF F_1 HYBRID PLANTS OBTAINED FROM RESCUED EMBRYOS 41

TABLE 2-5. FLOWER COLOUR AND LEAF MORPHOLOGY OF THE F_1 HYBRID PLANTS DERIVED FROM RECIPROCAL INTERSPECIFIC CROSSES BETWEEN *B. NAPUS* AND *B. OLERACEA* VAR. *ALBOGLABRA* 43

TABLE 2-6. AGRONOMIC TRAITS OF THE F_1 HYBRID PLANTS (MEAN ± SD) DERIVED FROM RECIPROCAL INTERSPECIFIC CROSSES BETWEEN *B. NAPUS* AND *B. OLERACEA* VAR. *ALBOGLABRA* 44

TABLE 2-7. NUMBER OF FERTILIZED OVULES PER SILIQUE FROM F_1 HYBRID PLANTS POLLINATED WITH *B. OLERACEA* VAR. *ALBOGLABRA* IN A GROWTH CHAMBER 46

TABLE 2-8. EFFICIENCY OF *IN VITRO* OVULE CULTURE TECHNIQUE VS. *IN VIVO* SEED SET FOR THE PRODUCTION OF INTERSPECIFIC BC_1 HYBRIDS IN A GROWTH CHAMBER 46

TABLE 3-1. EXPECTED ERUCIC ACID PHENOTYPES OF THE BC_1S_1 SEED GENOTYPES WITH ZERO, ONE, OR TWO A-GENOME CHROMOSOMES CARRYING THE ZERO ERUCIC ACID ALLELE (A^0) 61

TABLE 3-2. FLOWER COLOUR AND PLANT TYPE OF THE (*B. NAPUS* X *B. ALBOGLABRA*) X *B. ALBOGLABRA* BC_1 HYBRID PLANTS 62

TABLE 3-3. POLLEN FERTILITY IN 10 BC_1 PLANTS OF (*B. NAPUS* X *B. ALBOGLABRA*) X *B. ALBOGLABRA* 63

TABLE 3-4. SEED SET IN BC_1 PLANTS (=BC_1S_1 SEEDS) FROM MANUAL SELF-POLLINATION AND SELF-POLLINATION BY BAG ISOLATION 63

TABLE 3-5. SEGREGATION FOR PETAL COLOUR AND ERUCIC ACID CONTENT IN BC_1S_1 POPULATION DERIVED FROM SELF POLLINATIONS OF (*B. NAPUS* X *B. OLERACEA* VAR. *ALBOGLABRA*) X *B. OLERACEA* VAR. *ALBOGLABRA* BC_1 PLANTS 66

TABLE A-1. MICROSATELLITE PRIMERS USED FOR EVALUATION OF THE C-GENOME OF *B. NAPUS* AND *B. OLERACEA* VAR. *ALBOGLABRA* FOR POLYMORPHISM 73

List of Figures

FIGURE 1-1. AREA OF THE FOUR MOST IMPORTANT AGRICULTURAL CROPS IN CANADA, 2003 TO 2007 (STATISTICS CANADA 2007)..... 2

FIGURE 1-2. SIMPLIFIED DIAGRAM REPRESENTING TWO BIOSYNTHETIC PATHWAYS USING OLEIC ACID AS A SUBSTRATE..... 12

FIGURE 1-3. INTERRELATIONSHIPS BETWEEN DIPLOID AND TETRAPLOID *BRASSICA* SPECIES, AS SET FORTH BY U (1935). THESE SIX SPECIES ARE COMMONLY KNOWN TODAY AS THE 'CROP BRASSICAS'..... 16

FIGURE 2-1. AVERAGE LENGTH OF SILIQUES OF *B. NAPUS* X *B. OLERACEA* VAR. *ALBOGLABRA* RECIPROCAL INTERSPECIFIC CROSSES UNDER TWO TEMPERATURE CONDITIONS AND HARVESTED AT SIX DIFFERENT AGES..... 37

FIGURE 2-2. NUMBER OF FERTILIZED OVULES PER POLLINATION FOLLOWING RECIPROCAL INTERSPECIFIC CROSSES BETWEEN *BRASSICA NAPUS* AND *B. OLERACEA* VAR. *ALBOGLABRA* AND UNDER TWO TEMPERATURE CONDITIONS. AVERAGE VALUES OF HI-Q X *B. ALBOGLABRA* AND A01-104NA X *B. ALBOGLABRA* (*B. NAPUS* X *B. OLERACEA*); *B. ALBOGLABRA* X HI-Q AND *B. ALBOGLABRA* X A01-104NA (*B. OLERACEA* X *B. NAPUS*) ARE GIVEN..... 38

FIGURE 2-3. HYBRID EMBRYO YIELD FOR THE RECIPROCAL CROSSES OF *B. NAPUS* AND *B. OLERACEA* VAR. *ALBOGLABRA* FROM TWO TEMPERATURE CONDITIONS AND SILIQUES HARVESTED AT SIX DIFFERENT DATES. AVERAGE VALUES OF HI-Q AND A01-104NA PRESENTED AS *B. NAPUS*..... 40

FIGURE 2-4. MICROSATELLITE (SSR) MARKER AMPLIFICATION PATTERNS OF THE PARENTAL SPECIES AND THEIR INTERSPECIFIC F_1 HYBRIDS OF HI-Q X *B. OLERACEA* AND *B. OLERACEA* X HI-Q CROSSES. PARENTS ARE SHOWN WITH ARROWS: HI-Q (LEFT), *B. ALBOGLABRA* (RIGHT)..... 42

FIGURE 2-5. LEAF MORPHOLOGY OF *B. OLERACEA* VAR. *ALBOGLABRA* (LEFT), *B. NAPUS* (A01-104NA) (RIGHT), AND THEIR INTERSPECIFIC F_1 HYBRID PLANT (CENTER)..... 44

FIGURE 3-1. EXPECTED GENOTYPIC FREQUENCIES IN THE BC_1S_1 GENERATION IN A DIPLOID *B. OLERACEA* GENOTYPE SEGREGATING FOR C-GENOME ERUCIC ACID ALLELES..... 61

FIGURE 3-2. DISTRIBUTION OF THE BC_1S_1 SEEDS FOR ERUCIC ACID CONTENT (N=109)..... 65

FIGURE 3-3. OBSERVED NUMBER OF BC_1S_1 SEEDS FOR THREE DIFFERENT ERUCIC ACID CLASSES COMPARED TO THE NUMBER OF SEEDS TO BE EXPECTED FROM NORMAL DISOMIC SEGREGATION BASED ON ONLY THE C-GENOME ERUCIC ACID ALLELES. BC_1S_1 SEEDS DERIVED FROM SELF-POLLINATION OF (*B. NAPUS* X *B. OLERACEA* VAR. *ALBOGLABRA*) X *B. OLERACEA* VAR. *ALBOGLABRA* HYBRID PLANTS..... 65

Symbols and Abbreviations

± – Plus/minus
°C – Degrees Celsius
µE – Microeinstein
µL – Microliter
µmol/g – Micromoles per gram
χ^2 – Chi-square test statistic

A^0 – Recessive erucic acid allele from *B. napus* A-genome

BC_1 – First backcross generation
BC_1S_1 – First self-pollinated generation after backcross

C^+ – High erucic acid allele from *B. oleracea* C-genome
C^0 – Recessive erucic acid allele from *B. napus* C-genome
C_2 – Molecule consisting of two carbon atoms
cm – Centimeter
CQ – Canola quality
cv. – cultivar
Cx:x – Fatty acid (x carbon atoms and x double bond(s))

DAP – Days after pollination
ddH_2O – Double distilled water
DH – Doubled haploid
DNA – Deoxyribose nucleic acid
dNTP – Deoxynucleotide triphosphate

EtBr – Ethidium bromide

F_1 – First filial generation
FAE1 – Fatty acid elongase gene
F_x – xth filial generation

g - Gram
GDP – Gross domestic product

h – hour
HOLL – High oleic, low linolenic canola

L – Liter

m^2 – Square meter
MAS – Marker-assisted selection
mg – Milligram
$MgCl_2$ – Magnesium chloride
min – Minute
mL – Milliliter
mm – Millimeter
mM – Millimole
MS – Murashige and Skoog
mV – Millivolt

n – Haploid number of chromosomes
ng – Nanogram
NN – Nitsch and Nitsch
No. – Number

P – Probability
PCR – Polymerase chain reaction
pH – Measure of acidity/alkalinity of a solution
poll. – Pollinations

QTL – Quantitative trait loci

rpm – Revolutions per minute

s – Second
SD – Standard deviation
ssp. – Subspecies
SSR – Simple sequence repeat

t – Test statistic for t-test
Taq (polymerase) – Polymerase from the bacterial species *Thermus aquaticus*
Temp. – Temperature

UV – Ultraviolet (light)

V – Volt
var. – Variety

w/v – Weight to volume ratio

x – Basic chromosome number

Chapter 1

Literature review

1.1 Introduction

Rapeseed (*Brassica napus* and *B. rapa*) is one of the most important oilseed crops in the world (Table 1-1), and it is most commonly cultivated in areas with temperate climates. Winter forms of rapeseed *B. napus* are commonly grown in Europe, semi-winter types in China, and spring types in Canada, Australia, and Northern Europe. Most rapeseed varieties cultivated for edible purposes are of 'double low' or 'canola' quality. Canola quality rapeseed varieties are defined as having less than two percent erucic acid in the oil and less than 30 micromoles glucosinolates per gram of the solid component of the seed. Canola is the most important oilseed crop in Canada and it is second only to wheat in total seeded area. Approximately 6.0 million hectares were seeded in 2007 (Figure 1-1), almost all of which was grown in the Western Canadian Prairie Provinces. Productivity of this crop has steadily increased, averaging 1.32 t ha^{-1} in the five-year period of 1992 to 1996, 1.42 t ha^{-1} during 1997 to 2001, and 1.54 t ha^{-1} in 2002 to 2006 (Statistics Canada 2007). The canola industry plays an important role in the Canadian economy. The revenue generated from each stage of the chain of production of canola (e.g. earnings by producers, value of oil and meal, crushing/refining, multiplier effect of value added components) sums to a total of more than $11 billion annually, which accounts for 1% of the total GDP (Mark Goodwin Consulting Ltd. 2006). The Canola Council of Canada, a non-profit association that represents the entire canola industry in Canada, has set ambitious goals to increase total production from 9 million tonnes in 2006 to 15 million tonnes by 2015. In order to achieve this goal, productivity must

increase. Cultivable area for canola production is limited, especially due to a recommended four-year rotation to avoid the onset of serious disease and pests.

Table 1-1. Oilseed production (in 1,000 tonnes) in selected countries for 2004 (FAO Statistical Yearbook 2005)

Country	Soybeans	Sunflower seed	Rapeseed	Cotton seed
Argentina	31,500	3,100	20	190
Australia	74	58	1,549	912
Brazil	49,793	200	60	2,246
Canada	3,048	54	7,728	0
China	17,600	1,750	13,182	12,640
Germany	1	70	5,277	0
India	7,500	1,300	6,200	5,130
Mexico	76	1	14	200
Pakistan	10	404	401	4,853
Sweden	0	0	228	0
USA	85,013	930	613	7,477
World	206,408	26,466	46,171	43,334

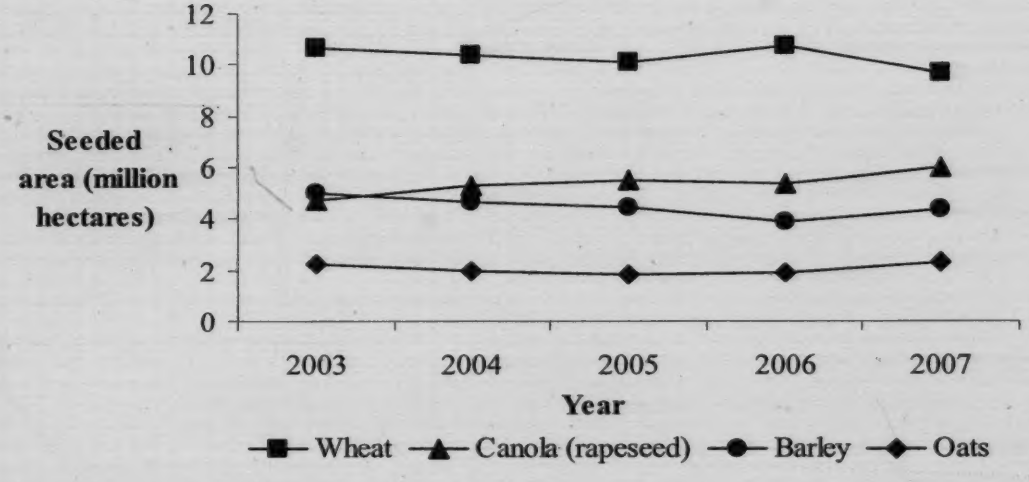

Figure 1-1. Area of the four most important agricultural crops in Canada, 2003 to 2007 (Statistics Canada 2007).

1.2 Genetic diversity in *B. napus*

Canola is comprised of two species in Canada: *B. napus* L. and *B. rapa* L. (synonym *B. campestris* L.). Of these two species, *B. napus* currently dominates the industry with more than 90% of the seeded area. Germplasm improvement in *B. napus* is hindered because this species has limited genetic diversity, particularly in the spring types grown in Canada (Hasan et al. 2006, Qian et al. 2006). New genetic variation conferring increased seed yield and disease resistance is essential for the development of improved cultivars. In the case of hybrid cultivars, use of genetically diverse parents may also provide greater levels of heterosis for seed yield and other agronomic traits (Riaz et al. 2001). Thus, canola producers in Canada could greatly benefit from the increased genetic diversity because of its potential for continued improvement in this crop.

Three primary factors apparently resulted in the narrow genetic base in the currently available germplasms of *B. napus*: (i) it was derived from a few spontaneous hybridizations that occurred in a limited geographical area, (ii) domestication occurred only 400 to 500 years ago (Gómez-Campo and Prakash 1999), and (iii) the mutants with specific oil and quality traits were used intensively in repeated cycles of breeding (Seyis et al. 2003b, Hasan et al. 2006). Due to the economic importance of *B. napus*, research on broadening the genetic diversity in this species has increased.

Genetic variation has been studied in *B. napus* germplasm as well as in its allied species to identify the important sources of diversity. A variety of methods have been applied to group *Brassica* species based on genetic diversity. Some of the researchers used agronomic characteristics (Hu et al. 2007) and isozymes (Becker et al. 1995, Yu et al. 2005); however, the most commonly used method to assess genetic diversity is the use

of DNA molecular markers. These markers are based on polymorphic segments of plant DNA that provide useful information on the genetic relationship between different individuals. The DNA markers are also commonly used in breeding programs for marker-assisted selection (MAS) and in phylogenetic studies (Snowdon and Friedt 2004). The invention and subsequent development of polymerase chain reaction (PCR) techniques (Mullis and Faloona 1987) played an important role in facilitating the widespread use of molecular markers in research and breeding programs. The PCR method allows researchers to amplify specific regions of genomic DNA, located between two primer sites, exponentially in short time (<5 min $cycle^{-1}$); thus a very small quantity of leaf sample/DNA is required in contrast to the non-PCR based method, e.g. restriction fragment length polymorphism (RFLP). Examples of commonly used PCR-based molecular markers in studies on genetic diversity include simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP), and randomly amplified polymorphic DNA (RAPD).

Several approaches have been used to increase genetic diversity in the rapeseed species *B. napus*. One of the approaches is to utilize the diversity from winter and semi-winter types of this species, which were found to be genetically distinct from spring type *B. napus* (Diers and Osborn 1994, Becker et al. 1995, Plieske and Struss 2001, Relf-Eckstein and Rakow 2003, Quijada et al. 2004, Udall et al. 2004, Qian et al. 2007). Spring oilseed types have been found to have low levels of diversity compared to other types, such as winter oilseed, fodder, vegetable, and interspecific-derived types (Hasan et al. 2006, Qian et al. 2006).

Brassica napus cultivars and lines from different geographic origins may also be a source for increased variation in Canadian canola. Hu et al. (2003, 2007) compared several accessions of rapeseed/canola from Europe and China based on agronomic characters and RAPD analysis, and reported that most of the accessions from Europe were genetically distinct from most of the accessions from China. Diers and Osborn (1994), and Zhao and Becker (1998), came to similar conclusions based on RFLP and isozyme markers that the Chinese gene pool is genetically distinct from other gene pools. These gene pools could also be used for improvement of specific traits of this crop, e.g. high seed oil content, as these two gene pools were found to carry different alleles for this trait (Zhao et al. 2005).

B. napus (AACC, 2n=38) is an amphidiploid, resulting from spontaneous hybridizations in nature between the diploid species *B. rapa* (AA, 2n=20) and *B. oleracea* (CC, 2n=18) (U 1935, Song and Osborn 1992). Phylogenetic studies based on molecular markers suggests that *B. napus* is genetically distinct from its parental species (Song et al. 1988, Thormann et al. 1994). Therefore, these two diploid species would serve as a reservoir of genetic diversity for improving *B. napus*. Introgression of genetic diversity can be done by (i) crossing *B. napus* with either of its progenitor species, or (ii) through resynthesis of *B. napus* from its progenitor species.

Interspecific crosses of *B. napus* with its two progenitor species have typically been done to introgress specific traits from one species to the other. *Brassica rapa* has often been a good choice by several researchers for crossing with *B. napus* for introducing desired traits (Johnston 1974, Scarth et al. 1992). The good cross-compatibility with *B. napus*, as well as good fertility in the interspecific offspring (Bing et al. 1996), are two

advantages of using *B. rapa* as these factors play a vital role in successful introgression of trait(s) in wide crosses. Qian et al. (2006) compared genetic diversity in *B. napus* x *B. rapa* interspecific cross derived *B. napus* lines with commonly available spring, winter, and semi-winter *B. napus* lines, and reported that the interspecific cross derived lines had the highest genetic diversity, and could be used to diversify the *B. napus* germplasm worldwide. Miller (2001) studied the performance of doubled haploid (DH) lines derived from *B. napus* x *B. rapa* crosses, and concluded that simultaneous introduction of many favourable alleles into *B. napus* is difficult. Therefore, additional cycle(s) of breeding would be required to combine the introgressed diversity for further improvement of this crop.

The resynthesis of *B. napus* by crossing *B. rapa* and *B. oleracea* has been considered to be an important strategy for broadening the genetic diversity in *B. napus* (Engqvist and Becker 1994, Becker et al. 1995, Seyis et al. 2003b). Wide genetic variability in the progenitor species will be required to generate great variability in the resynthesized rapeseed lines. Unfortunately, most resynthesized *B. napus* lines possess agronomically inferior traits such as late maturity and low seed yield (Kräling 1987). Therefore, these materials must be improved by crossing with elite breeding lines (Kräling 1987) or through recurrent selection without any backcrossing to breeding materials (Engqvist and Becker 1994). Seyis et al. (2003a) crossed resynthesized *B. napus* lines with male sterile breeding lines, and found higher yield potential in the resulting hybrids.

One of the most important direct benefits of broadening genetic diversity in *B. napus* would be its use in hybrid breeding. Increased genetic distance between the

parents often results in increased levels of heterosis in the hybrids. Heterosis can be obtained for many agronomic traits including seed yield, oil content, and total biomass. Diers et al. (1996) and Riaz et al. (2001) reported a significant correlation between genetic distance based on molecular markers and heterosis for seed yield. However, use of molecular markers alone to predict hybrid performance could be somewhat unreliable (Yu et al. 2005).

1.3 Canola meal and oil quality

Two products are derived from canola seed – the oil which is primarily used for human consumption and the meal which is considered a by-product and sold as a feed supplement. Oil-free canola seed meal contains 39 to 44% protein composed of favourable amino acids. However, the meal contains 12% crude fiber, which limits its energy value. Therefore, seed or meal with low fiber content is desirable. Seed coat colour significantly influences the protein/fiber ratio in the seed meal. Yellow-seeded canola generally has thinner seed coat and has been shown to have, on average, higher levels of protein, lower levels of fiber, and higher oil content than their black-seeded counterparts (Simbaya et al. 1995, Rahman et al. 2001). These attributes apparently are due to a higher contribution of embryo to the seed in the yellow-seeded type (Stringam et al. 1974). Therefore, development of the yellow-seeded type has been an important breeding objective and several researchers have successfully developed yellow-seeded *B. napus* lines (Rashid et al. 1994, Rahman 2001). The development of elite, highly adapted yellow-seeded cultivars is still in progress (Relf-Eckstein et al. 2007).

Oil is the most important component of canola, accounting for 80% of the revenue. As such, an important breeding objective has always been to increase the seed oil content.

During the five-year period from 1997 to 2001, the average oil content for Canadian canola was 43.0%; while from 2002 to 2006 the average oil content reached 43.3%, i.e. 0.3% greater (DeClercq 2006). The canola industry in Canada has set the target to achieve 45% oil content by 2015. An increasing worldwide interest in renewable fuels, such as biodiesel, has placed added importance on developing varieties that produce larger quantities of oil per unit area of land.

Oil quality, which is determined by its fatty acid composition, can be equally or more important than seed yield. Fatty acids are organic molecules consisting of a hydrocarbon chain and a terminal carboxyl group, and are commonly found in fats and oils. The fatty acid profile of the oleiferous plant species determines their usefulness for industrial applications or human consumption. The competitive advantage of canola oil in today's world market is largely due to its desirable fatty acid profile.

All canola cultivars grown in Canada that are destined for human consumption contain <0.2% erucic acid in oil. Erucic acid is a 22-carbon monounsaturated fatty acid considered undesirable for edible purposes. Members of the family *Brassicaceae* (the mustard family) are characterized by a high level of erucic acid in the oil. Some of the earliest licensed rapeseed varieties in Canada, such as Echo (a *B. rapa* variety; released in 1964) possessed ca. 23.5% erucic acid and Target (a *B. napus* variety; released in 1966) had ca. 40.0% erucic acid. Experiments on the nutritional value of rapeseed oil conducted on rats indicated that erucic acid in the oil caused inhibited growth (Thomasson and Boldingh 1955, Beare et al. 1959) and increased concentrations of cholesterol in the adrenal cortex, liver, and feces (Beare 1957). These studies questioned the safety of rapeseed oil, and a general conclusion of its undesirability for human consumption was

made. Since then, development of erucic acid-free cultivars has been of prime importance in the breeding programs in Canada and Europe. The world's first erucic acid-free strain of *B. napus* was isolated in Winnipeg by Stefansson et al. (1961) from the German *B. napus* cultivar 'Liho'; and the first erucic acid-free strain of *B. rapa* was isolated in Saskatoon by Downey (1964). These naturally occurring mutants/strains were extensively used in breeding programs in North America and Europe for the development of zero-erucic acid rapeseed cultivars (Stefansson and Hougen 1964).

Canola oil contains a low level of saturated fatty acids (7%), the lowest among all vegetable oils (POS Pilot Plant Corporation 1994). A high intake of saturated fats has been found to increase the levels of low-density lipoprotein cholesterol ("bad" cholesterol) in the blood, which is associated with an increased risk of coronary heart disease (Barr et al. 1992). This oil contains 58 to 60% oleic acid (C18:1), a monounsaturated fatty acid found to be effective in lowering low-density lipoprotein cholesterol (Mattson and Grundy 1985). Typical canola oil also has a favourable balance of polyunsaturated fatty acids, 21% linoleic acid (C18:2), and 11% alpha-linolenic acid (C18:3) (POS Pilot Plant Corporation 1994). Both are considered essential fatty acids, as they cannot be synthesized by the human body and must be obtained from the diet. Alpha-linolenic acid is an omega-3 fatty acid, a group of fatty acids associated with decreased risk of coronary artery disease, myocardial infarction, and stroke (Psota et al. 2006).

Other fatty acid profiles in canola oil have been developed for food or non-food applications. High oleic, low linolenic (HOLL) (>75% oleic, <3% linolenic) canola oils are favoured by the food industries as they are less prone to oxidation and more

thermostable under deep frying conditions (Orthoefer 2005). In the manufacture of margarine and shortening, this type of oil does not need hydrogenation – a process that creates *trans* fats. Oils high in erucic acid content are desirable for industrial applications, particularly for the production of erucamide, a slip agent used in the manufacture of plastic bags (Scarth and Tang 2006).

1.3.1 Genetics of erucic acid

Harvey and Downey (1964) studied the inheritance of erucic acid in seed oil of *B. napus*, and suggested that: (i) two gene loci control this trait, (ii) each allele acts in an additive, i.e. non-dominant manner, and (iii) erucic acid content is embryonically rather than maternally controlled. These observations were confirmed by Kondra and Stefansson (1965). Dorrell and Downey (1964) came to similar conclusions with *B. rapa*, but found that only one gene locus controlled erucic acid in this species. These findings led to the logical conclusion that *B. napus* inherited one erucic acid gene from each of its progenitor species; and this has been further confirmed by Chen and Heneen (1989). Subsequent studies on the amphidiploid species *B. juncea* (AABB) (Kirk and Hurlstone 1983) and *B. carinata* (BBCC) (Getinet et al. 1997) also revealed that two gene loci controlled erucic acid in these species. Rahman et al. (1994) reported partial dominance of the high erucic acid allele of Yellow Sarson *B. rapa* (>50% erucic acid) over the zero-erucic acid allele. Several different alleles of the erucic acid gene, which act in an additive or partially dominant manner, have been reported in the literature (Table 1-2).

Table 1-2. Selected erucic acid alleles described in the literature; reviewed by Getinet et al. (1997). Gene nomenclature was proposed by Krzymanski and Downey (1969)

Allele	Erucic acid contribution (%)	Species
e	0	Zero erucic *B. napus*[z] and *B. rapa*[y]
E^a	10	*B. napus* cv. 'Golden'[x] and 'Nugget'[w]; *B. carinata*[v]
E^b	12-15	*B. rapa* landrace 'Polish'[u]
E^c	30	*B. rapa* var. 'Yellow Sarson'[u]
E^d	3.5	*B. napus* cv. 'Bronowski'[t]

References: [z]Stefansson et al. (1961), [y]Downey (1964), [x]Harvey and Downey (1964), [w]Kondra and Stefansson (1965), [v]Getinet et al. (1997), [u]Dorrell and Downey (1964), [t]Krzymanski and Downey (1969).

Oleic acid (C18:1) is used as a substrate in two biosynthetic pathways. It is desaturated to form linoleic and linolenic acid, or chain elongation occurs in which C_2 units are added to form eicosenoic and erucic acid (Figure 1-2). A mutation in the fatty acid elongation gene (*FAE1*) results in the zero-erucic acid phenotype (Barret et al. 1998, Fourmann et al. 1998). The *FAE1* gene encodes β-keto-acyl-CoA synthase, a coenzyme carrying a two carbon atom group, which catalyzes the first step in the oleic acid elongation pathway (Figure 1-2). Allelic variation in this gene can result in varying levels of enzymatic activity, thus influencing overall erucic acid production in the seed.

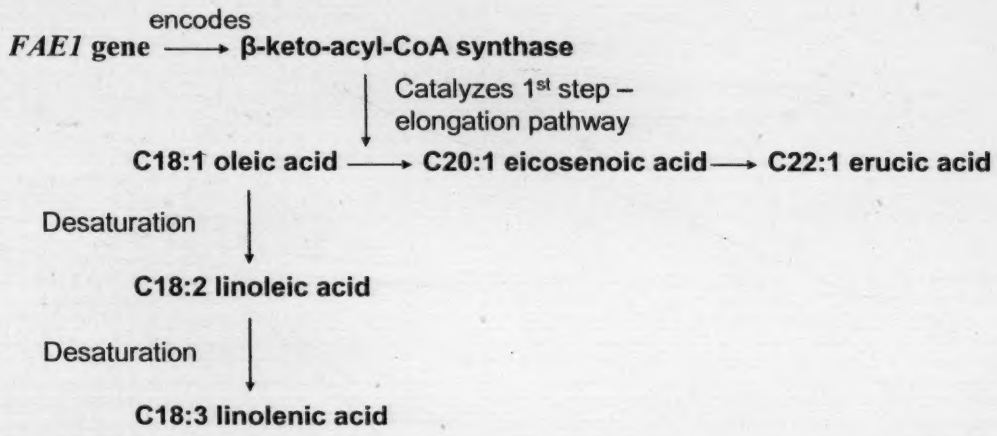

Figure 1-2. Simplified diagram representing two biosynthetic pathways using oleic acid as a substrate.

Genotype x environment interactions have also been found to have an influence on erucic acid inheritance (Shi et al. 2003). Harvey and Downey (1964) observed an environmental influence on erucic acid content and suggested this influence was greater on individuals capable of producing large amounts of this fatty acid. Kirk and Hurlstone (1983) and Getinet et al. (1997) also found higher variability in genotypes capable of producing greater amounts of erucic acid in *B. juncea* and *B. carinata*. Erucic acid content was found to be negatively correlated with moisture and temperature (Craig 1961). Rahman et al. (1994) crossed two *B. rapa* varieties, 'Yellow Sarson' (54% erucic) and 'Tobin' (zero-erucic), and reported that segregation distortion for this trait can occur due to growth condition as well as when the parents are genetically very diverse.

1.3.2 Genetics of seed glucosinolate

Canola quality seed is defined as having less than 30 micromoles of one or any combination of 3-butenyl (gluconapin), 4-pentenyl (glucobrassicanapin), 2-hydroxy-3-butenyl (progoitrin), and 2-hydroxy-4-pentenyl (napoleiferin) glucosinolates per gram of

air-dry oil-free solid. These glucosinolates belong to a group known as aliphatic glucosinolates derived from the precursor amino acid methionine. About 30 different glucosinolates occur in the *Brassica* species (Sørenson 1990), and these species have different profiles of glucosinolates (Valesco and Becker 2000). Some of these glucosinolates, e.g. sinigrin, impart an adverse effect on the taste and smell of animal feed; while other glucosinolates, e.g. progoitrin (goitrogenic), are antinutritional and decrease the feeding value of this high-protein meal (Bjerg et al. 1989). Therefore, breeding programs are focused on reducing the levels of total and/or specific glucosinolates for increasing value of this seed meal.

Finlayson et al. (1973) reported the first low glucosinolate (total glucosinolate 10 μmol g^{-1} seed) genetic variation found in the *B. napus* Polish fodder variety 'Bronowski'. Alleles from this variety have been introduced into oilseed *Brassica*, and virtually all double-low cultivars used today carry the 'Bronowski' alleles. Several studies have been done to understand the genetic control of *Brassica* seed glucosinolate production. These estimates are based on the known biosynthetic steps for glucosinolate synthesis; as well as specific plant material with varying glucosinolate profiles used in these experiments. *B. napus* has a relatively uniform seed glucosinolate profile. Therefore, studies on the inheritance of glucosinolate have also been done by crossing of synthetic *B. napus* lines, derived from *B. rapa* and *B. oleracea* with varying profiles of glucosinolates, with conventional *B. napus* cultivars.

Magrath et al. (1993), based on crossing of resynthesized and natural *B. napus*, suggested that six unlinked loci determine the aliphatic glucosinolate content in *B. napus*. Interactions between the genes responsible for side chain elongation and side chain

modification results in a complex mixture and varying quantity of aliphatic glucosinolates. Rücker and Röbbelen (1994) reported that alkenyl (aliphatic) glucosinolate profiles in winter forms of *B. napus* are determined by four gene loci; while the total low glucosinolate content is due to four to five recessive genes in homozygous condition. They found that heritability of glucosinolate content is high. Similarly, Rahman et al. (2001) reported at least four gene loci to be involved in the control of total seed glucosinolate in spring type *B. napus*; and low glucosinolate is due to the presence of recessive alleles in the homozygous condition in all loci. Quantitative trait loci (QTL) studies have also been performed to identify the chromosomal regions associated with the inheritance of seed glucosinolate content. Uzunova et al. (1995), using doubled haploid (DH) populations derived from crosses between high and low glucosinolate winter-type *B. napus* lines, identified four QTLs for the total glucosinolate content. These QTLs explained 61% of the phenotypic variation in this population. Based on spring-type DH lines, Toroser et al. (1995) identified five QTLs for total glucosinolate which behaved in an additive fashion and explained 71% of the phenotypic variation in this population.

Thus, aliphatic seed glucosinolate content appears to be controlled by four to five gene loci and total low glucosinolate phenotype results from recessive alleles in all loci in homozygous condition. Little is currently known on the genetic control of indolyl and aromatic seed glucosinolates.

1.4 Wide hybridization in *Brassica*

The correct selection of parents is of utmost importance in all plant breeding programs. Wide hybridization is used when a gene or genes of interest are not available within the primary gene pool. Backcrossing to the well adapted recurrent parent is often

done to introgress the specific trait into a desirable genetic background. A good example of this is the transfer of disease resistance genes from one species to another. Rahman et al. (2007) successfully transferred blackleg (*Leptosphaeria maculans*) resistant genes from the B-genome of *B. carinata* (BBCC, 2n=34) into *B. napus* cultivar 'Westar' background. This was accomplished through interspecific crosses between these two species, followed by backcrossing to 'Westar'. Similarly, Plieske et al. (1998) obtained blackleg resistant recombinant lines through creating a trigenomic amphihaploid (ABC genome) and subsequently backcrossing this to the Canadian spring *B. napus* cultivar 'Andor'. Wide hybridization has also been used for a variety of other breeding objectives in *B. napus*, such as development of self-incompatible lines (Ripley and Beversdorf 2003, Rahman 2005), introgression of yellow seed colour genes (Rahman 2001), and improved earliness (Akbar 1989).

1.4.1 *Brassica* genome relationships

Traditionally, the species relationships in the genus *Brassica* have been postulated based on cytological evidence. This is a difficult task because of the small size of their chromosomes (Lydiate et al. 1993). Early cytological studies conducted by Morinaga (1934) and U (1935) demonstrated the interrelationships between the diploid and tetraploid *Brassica* species (Figure 1-3). The genomes of the diploid species were designated as 'A' for *B. rapa* (n=10), 'B' for *B. nigra* (n=8), and 'C' for *B. oleracea* (n=9). The amphidiploid species *B. napus* (AC genome, n=19), *B. juncea* (AB genome, n=18), and *B. carinata* (BC genome, n=17) are hypothesized to be the result of natural crossing between these diploid species. This has been proven through experimental synthesis of the amphidiploids from interspecific crosses between the diploid species

followed by chromosome doubling (U 1935, Frandsen 1943, Frandsen 1947, Olsson 1960). The synthetic amphidiploids are able to produce fertile offspring in crosses with natural amphidiploids of the same species.

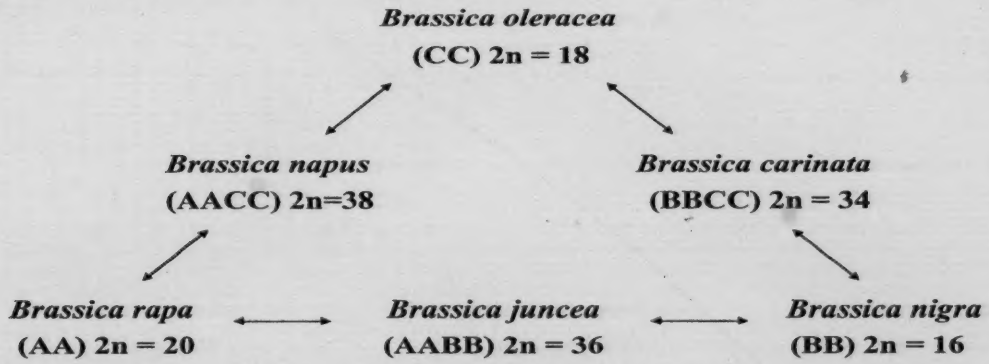

Figure 1-3. Interrelationships between diploid and tetraploid *Brassica* species, as set forth by U (1935). These six species are commonly known today as the 'crop brassicas'.

More recently, molecular markers have been applied for studying the genome relationships in *Brassica*. Song et al. (1988) used RFLPs to study the phylogenetic origins of these six species, and confirmed that the three amphidiploid species were indeed the result of hybridizations between the three diploid species. They suggested that the amphidiploid species, particularly *B. napus* and *B. juncea*, are of polyphyletic origin derived from different morphotypes of their diploid progenitors. They also provided evidence that the cultivated *B. napus* is closer to the C-genome than it is to the A-genome.

It is apparent that the diploid *Brassica* genomes have evolved from an ancient progenitor – however, the exact mechanism is still controversial. Based on cytological observation of the pachytene chromosomes, Röbbelen (1960) proposed that the diploid species arose from a progenitor species of chromosome number of six (x=6) through

addition or duplication of individual chromosomes. Lagercrantz and Lydiate (1996) performed a comparative analysis on the diploid species genomes using a common set of RFLPs, and found evidence that the diploid *Brassica* species result from genome-wide triplication of a hexaploid ancestor because many loci are present in three copies in these species. These researchers suggested that the different chromosome numbers (8, 9, and 10) in the diploid species are the result of chromosome fission and fusion events, rather than addition/deletion of entire chromosomes. Lukens et al. (2004) found strong evidence of genome replication and rearrangement, but little evidence for genome triplication. Two lineages have been suggested for diploid *Brassica* species based on analyses of chloroplast genomes – a *B. rapa*/*B. oleracea* lineage and a *B. nigra* lineage (Warwick and Black 1991). These studies suggest a closer relationship between the A- and C- genomes than either has with the B-genome.

1.4.2 Hybridization barriers

The fundamental goal in a plant breeding program is to develop cultivars with desirable combinations of genes. Use of wide crosses is often very challenging, but can be extremely rewarding in the long-term perspective (Hadley and Openshaw 1980). Hybridization barriers often exist between species that prevent the formation of viable offspring. These barriers can be broadly classified into two categories: pre-fertilization and post-fertilization barriers. In pre-fertilization barriers, fertilization between sperm and egg from two species is prevented. Geographical, ecological, and temporal isolation, as well as the prevention of fertilization after pollination, are examples of pre-fertilization barriers.

Hybridization between *Brassica* species is often restricted by post-fertilization barriers. This includes degeneration of hybrid embryos, inviable or sterile F_1 plants, and hybrid breakdown in subsequent generations. Ayotte et al. (1987) performed interspecific hybridizations between *B. napus* and *B. oleracea*, and hypothesized that failure of endosperm development was primarily responsible for embryo degeneration. They found that pollen tube growth and fertilization occur frequently in this interspecific cross; however, without any seed set. *In vitro* culture of these interspecific hybrid embryos on artificial medium produced viable offspring, suggesting their ability to survive despite abnormal genetic constitution. The size of the ovules in this interspecific cross was smaller than ovules from intraspecific *B. napus* crosses; and the interspecific endosperm was gelatinous whereas it was a viscous liquid in the *B. napus* intraspecific cross. Therefore, *in vitro* embryo rescue techniques are being suggested to improve the rate of hybrid production in interspecific crosses.

1.4.3 Embryo rescue techniques

Post-fertilization cross incompatibility barriers can often be overcome using different embryo rescue techniques, viz. ovary, ovule, and embryo culture. These techniques allow breeders to obtain hybrid plants with greater efficiency than obtaining F_1 hybrids from seeds. The choice of the technique and its effectiveness depends on the genotype of the parents involved in the cross, particularly the maternal genotype. Takeshita et al. (1980) investigated the efficiency of hybrid production for various interspecific crosses in *Brassica* and *Raphanus* using ovary, ovule, and embryo culture. They found that ovule culture was superior to ovary and embryo culture when *B. oleracea* was the female parent. Lu et al. (2001) applied an ovule culture technique in

interspecific crosses between *B. oleracea* and *B. rapa,* and obtained a larger number of hybrids using *B. rapa* as female compared to *B. oleracea* as female. Hadley and Openshaw (1980) made a general suggestion that crosses are more successful while using species with a higher number of chromosomes as the female parent.

The age of silique, following cross pollination, for rescue of embryos and culture media greatly influence the efficiency of hybrid production (Table 1-3). Rahman (2004) reported that using *B. oleracea* as female and *B. rapa* or *B. carinata* as male, the greatest efficiency of embryo culture technique was obtained when embryos were rescued at 24 to 28 days after pollination. He also reported that the hybrid embryos could not be rescued at 16 days after pollination due to their smaller size, or 40 days after pollination as they become degenerated. Zhang et al. (2004) tested four combinations of ovary culture media in interspecific crossings using a single genotype of *B. oleracea* as female and three genotypes of *B. rapa* as male, and found different rates of seed production per ovary within the same cross, depending on the culture medium used. They concluded that the culture medium is crucial for efficient production of interspecific hybrids through application of the ovary culture technique.

Table 1-3. Embryo rescue techniques applied for interspecific crosses between *B. napus* and *B. oleracea*

Female	Method of embryo rescue	DAP[z]	Media used
B. napus[y]	Ovule culture	8	NN (1967) medium supplemented with casein hydrolysate, glutamine, and sucrose
B. napus & *B. oleracea*[x]	Embryo culture	22	Inorganic salts from MS (1962) medium, amino acids and vitamins from Jensen's (1976) C17 medium, supplemented with citric acid, tri-potassium citrate, and sucrose
B. napus[w]	Embryo culture	12 to 17	Monnier's (1973) embryo culture medium
B. oleracea[v]	Ovule culture	10 to 20	White's (1963) agar medium supplemented with coconut milk, naphthaleneacetic acid, and kinetin

[z]DAP = Days after pollination.

References: [y]Ripley and Beversdorf (2003), [x]Quazi (1988), [w]Ayotte et al. (1987), [v]Takeshita et al. (1980).

1.5 Research objectives

The long-term objective of this research project is to develop a canola quality *B. oleracea* line from interspecific crossing of *B. napus* and *B. oleracea* var. *alboglabra*; and to utilize this line in the resynthesis of canola quality *B. napus* lines for broadening the genetic diversity in this species. However, interspecific crosses between *B. napus* and *B. oleracea* are known to be quite difficult and the rate of hybrid production is generally low. Therefore, the present study was focused to identify the optimal growth condition (temperature) and age of siliques for efficient production of hybrid plants from reciprocal interspecific crosses of *B. oleracea* var. *alboglabra* x *B. napus* through application of

ovule culture technique. Furthermore, the efficiency of production of BC_1 hybrids through ovule culture was investigated. The inheritance of erucic acid content in self-pollinated progeny of the BC_1 plants, i.e. BC_1S_1 seeds, was also investigated to select erucic acid-free *B. oleracea* var. *alboglabra*.

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Chapter 2

Effects of temperature and age of siliques on hybrid embryo yield from interspecific crosses between *Brassica napus* and *B. oleracea* var. *alboglabra*

2.1 Introduction

Brassica napus (AACC genome, 2n=38) is an amphidiploid species resulting from hybridizations in nature between *B. rapa* (AA genome, 2n=20) and *B. oleracea* (CC genome, 2n=18). The A- and C-genomes of these diploid progenitor species are known to be genetically distinct from the corresponding genomes of natural *B. napus* (Song et al. 1988, Thormann et al. 1994). The A-genome of *B. rapa* and the C-genome of *B. oleracea* carry important economic traits not normally found in the genome of *B. napus*. Several important traits have been introgressed from the diploid to the amphidiploid species. For example, self-incompatibility (Ripley and Beversdorf 2003) and cabbage aphid resistance (Quazi 1988) have been introgressed from *B. oleracea* to *B. napus*; and resistance to clubroot (*Plasmodiophora brassicae*) from *B. rapa* to *B. napus* (Johnston 1974, Gowers 1982). Some important traits have also been transferred from the amphidiploid to the diploid species. For example, triazine resistance (Ayotte et al. 1987) and clubroot resistance (Chiang et al. 1977) have been transferred from *B. napus* to *B. oleracea* and resistance to white rust (*Albugo candida*) race 7 has been transferred from *B. napus* to *B. rapa* (Scarth et al. 1992).

Interspecific hybrid plants are relatively easy to obtain from crosses between *B. napus* and *B. rapa* without application of cell and tissue culture technique (Jørgensen and Andersen 1994, Bing et al. 1996). However, the cross between *B. napus* and

B. oleracea is known to be quite difficult (Downey et al. 1980). Moreover, self-pollinated progeny from the cross between amphidiploid and diploid species often stabilizes into amphidiploid type. However, backcrossing of the hybrids with the diploid parent often yields diploid type plants in the segregating population (Zaman 1988, Rahman 2001). Therefore, it is difficult to introgress a trait from canola *B. napus* into *B. oleracea*; as an extensive effort is needed to obtain viable F_1 hybrids, as well as backcrossing the F_1 with *B. oleracea* to obtain BC_1 hybrids. To develop a sufficient number of interspecific offspring to work with, an efficient method of producing F_1 and BC_1 hybrids from these two species is needed.

Ovary, ovule, and embryo culture are commonly used as embryo rescue techniques in interspecific crosses in *Brassica* (Inomata 1993). Diederichsen and Sacristan (1994) evaluated the efficiency of ovule and embryo culture techniques for the production of reciprocal *B. rapa* x *B. oleracea* hybrids, and reported that ovule culture was more effective for rescue of hybrid embryos. Takeshita et al. (1980) investigated the efficiency of ovary, ovule, and embryo culture for the production of various *Brassica* and *Raphanus* interspecific hybrids, and found ovule culture to be superior to other methods when *B. oleracea* is used as female. They reported that young embryos which are not capable of surviving under embryo culture condition are capable of developing under ovule culture condition and yields hybrid plants. Kameya and Hinata (1970) suggested that composition of the ovule culture medium is important, and reported that ovules develop better on liquid medium than solid medium as long as they were not immersed.

The objective of this study was to identify the optimal growth condition (temperature) and time of rescue (days after pollination, DAP) of hybrid embryos for *in*

vitro ovule culture for the production of *B. napus* x *B. oleracea* var. *alboglabra* interspecific hybrids. The morphology of the F_1 plants and the efficiency of the application of embryo rescue technique for the production of BC_1 hybrids were also investigated.

2.2 Materials and methods

2.2.1 Parent material

Two canola quality *Brassica napus* L. doubled haploid lines, Hi-Q and A01-104NA, and one highly inbred (F_7) non-canola quality *Brassica oleracea* var. *alboglabra* Bailey (Chinese kale) line were used. Hi-Q is a spring type variety developed at the University of Alberta and registered as a conventional variety in 1999. A01-104NA is a spring type breeding line maintained in the University of Alberta canola breeding program. The *B. oleracea* var. *alboglabra* accession used in this study is a self-compatible spring type. Therefore, flowering time could easily be synchronized with *B. napus* in contrast to the other kale varieties such as *B. oleracea* var. *acephala* (marrowstem kale) and var. *fimbriata* (curly kale), which require up to eight weeks of vernalization to induce flowering.

2.2.2 Experimental design

Parental plants were grown in six-inch pots in SunGro® Sunshine Mix 4 (55 to 65% Canadian sphagnum peat moss, fine perlite, dolomitic limestone, gypsum, wetting agent). *B. oleracea* var. *alboglabra* was seeded 14 days prior to the *B. napus* parents for synchrony of flowering time. Plants were raised in two Conviron growth chambers set at 20°/15°C and 15°/10°C day/night temperatures with 16 h photoperiod. Photosynthetic

flux density in both cabinets was 450 μE (mV) m^{-2} s^{-1} at plant level. Under each growth condition, there were two replications consisting of eight *B. oleracea* plants and four plants of each of the two *B. napus* parents. Reciprocal crosses were made where individual female plants were pollinated with bulk pollen from four male plants. Unopened flower buds of the female parents, at the age of approximately one day prior to anthesis, were emasculated (anthers were removed) using forceps, and a generous amount of pollen was brushed on the stigma. Developing siliques at the age of 6 to 16 DAP were harvested with two-day intervals, and were used for rescue of hybrid embryos. *In vivo* hybrid seed set following cross-pollination was also recorded to compare the efficiency of the embryo rescue technique. Self-pollinations of the parents were also made for comparison with the cross-pollinated siliques.

2.2.3 Ovule culture

The ovule culture technique was applied for rescue of the hybrid embryos, and was performed in a laminar flow hood under aseptic conditions. Excised siliques were counted and measured. They were surface sterilized with a 7% (w/v) calcium hypochlorite [$Ca(OCl)_2$] solution for 10 min in sterile 50 mL conical tubes, and subsequently rinsed twice with distilled water. The siliques were longitudinally dissected using a sterile surgical blade and developing (fertilized) ovules were excised and counted. Developing ovules were determined to be those with a healthy and non-shrunken appearance. A small incision was made on the non-micropylar end of the developing ovules. These were floated on an approximately 5 mL liquid culture medium (Ripley and Beversdorf 2003) in a tissue culture petri dish (60 x 15 mm). The liquid medium was composed of Nitsch and Nitsch (1967) medium supplemented with 300 mg L^{-1} casein

hydrolysate, 200 mg L^{-1} glutamine; and concentration of sucrose was 13%. The medium was adjusted to pH 6.0, and filter-sterilized. Tissue culture petri dishes were sealed and placed on a shaker set at 60 rpm. After 2 to 3 weeks on the shaker, the number of developed embryos having an elongated root and shoot axis and conspicuous cotyledons (cotyledon stage) was recorded.

The embryos (cotyledon stage) were transferred from liquid culture medium to solid B_5 medium containing 0.1 mg L^{-1} GA_3, 20 g L^{-1} sucrose and 8 g L^{-1} agar (Coventry et al. 1988). Embryos were placed lightly on the solid medium in a petri dish (100 x 15 mm) and sealed. The petri dishes were initially placed at 4°C under lights (8 h photoperiod) for 2 to 4 days and were then moved to room temperature (22 to 25°C) and placed under lights (30 µE (mV) m^{-2} s^{-1} photosynthetic flux density at plant level; 12 h photoperiod). Embryos were kept on the solid medium for 3 to 4 weeks until fully germinated and roots were developed. These seedlings were transplanted to five-inch pots containing soil-free growth medium (Stringam 1971) and placed in a growth chamber (15°/10°C day/night temperature; 16 h photoperiod). The newly transplanted seedlings were covered with transparent plastic tubes for three to four days until hardened and deemed able to survive in the new environment. Leaf morphology of hybrid plants was recorded in the early bolting stage of plant development (canola growth stage 53, Canola Council of Canada 2003).

2.2.4 Confirmation of hybrids using SSR markers

Simple sequence repeat (SSR, microsatellite) molecular markers were used to confirm the hybrid nature of the F_1 plants. Young leaves from all putative F_1 hybrid plants and their parents were collected in 2 mL centrifuge tubes and immediately frozen

in liquid nitrogen. Samples were stored at -80°C until used. Genomic DNA was extracted using a SIGMA GenElute™ Plant Genomic DNA Miniprep Kit. The Molecular Probes™ Quant-It™ PicoGreen® dsDNA Assay Kit (Invitrogen) was used to quantify the concentration of DNA extracted from the leaf tissue; the concentration was adjusted to 10 ng/μL. For polymerase chain reaction (PCR) amplification, each reaction had a final volume of 20 μL and consisted of the following components: 10.04 μL ddH_2O, 2 μL 10X PCR buffer, 0.16 μL dNTP mixture (0.2 mM), 0.6 μL $MgCl_2$ (1.5 mM), 0.2 μL Taq polymerase (1U), 5 μL DNA template (50 ng), and 2 μL forward/reverse primers (0.5 μM). The PCR protocol, run on a GeneAmp® PCR System 9700, was as follows: 95°C/5 min, (95°C/1 min, 58°C/30 s, 72°C/1 min) x 30, 72°C/7 min, 4°C/hold. 6X DNA loading buffer was added (4 μL), and samples were run on 2% agarose gel (10 μL EtBr 100 mL^{-1}, added) at 200 V for 2 to 3 hours or until polymorphic bands were well separated. Amplified fragments were visualized using a FluorChem™ SP system (UV light).

2.2.5 Statistical analysis

Comparisons of mean fertilization rates of ovules from reciprocal crosses were done by paired t-test using proc TTEST in the SAS system (Statistical Analysis System, Inc. 1999). A two-tailed, one sample t-test was used to compare morphological data of the F_1 hybrids with their mid-parent value.

For yield of hybrid embryos, chi-square analysis was performed using proc CATMOD in the SAS system (Statistical Analysis System, Inc. 1999), with a null hypothesis that there was no difference in the number of hybrid embryos per pollination between the six different ages of the siliques (6 to 16 DAP), as well as under two temperature (20°/15°C and 15°/10°C) conditions. Due to the small number of embryos

obtained, data from the crosses involving Hi-Q and A01-104NA were pooled as '*B. napus*'.

2.3 Results

2.3.1 F_1 Hybrid

2.3.1.1 Silique set and development

Silique set was measured as the proportion of the developed siliques to the total number of pollinations. Under both temperature conditions, cross-pollinations of *B. napus* (as the female parent) with the pollen of *B. oleracea* resulted in a lower number of harvestable siliques (63.7%) compared to self-pollination of *B. napus* (87.5%). A similar trend was also noticed for cross-pollinations of *B. oleracea* compared to self-pollination of *B. oleracea* (65.6 vs. 83.3%) under the higher temperature condition. However, under the lower temperature condition (15°/10°C), similar silique set was observed due to cross- (87.5%) or self-pollination (83.3%).

The length of the interspecific cross-pollinated siliques continued to increase up to 10 to 12 days after pollination for all crosses (Figure 2-1). The interspecific cross-pollinated siliques were generally shorter than self-pollinated siliques. However, a striking difference in silique length was observed between self-pollinated and interspecific cross-pollinated siliques from *B. napus*. At 12 to 16 DAP, the self-pollinated siliques of *B. napus* were, on average, 47.8 mm longer at 20°/15°C and 34.5 mm longer at 15°/10°C than interspecific cross-pollinated siliques. In contrast, self-pollinated siliques of *B. oleracea* were only 2.8 mm (20°/15°C) and 5.9 mm (15°/10°C) longer than siliques from *B. oleracea* x *B. napus* crosses.

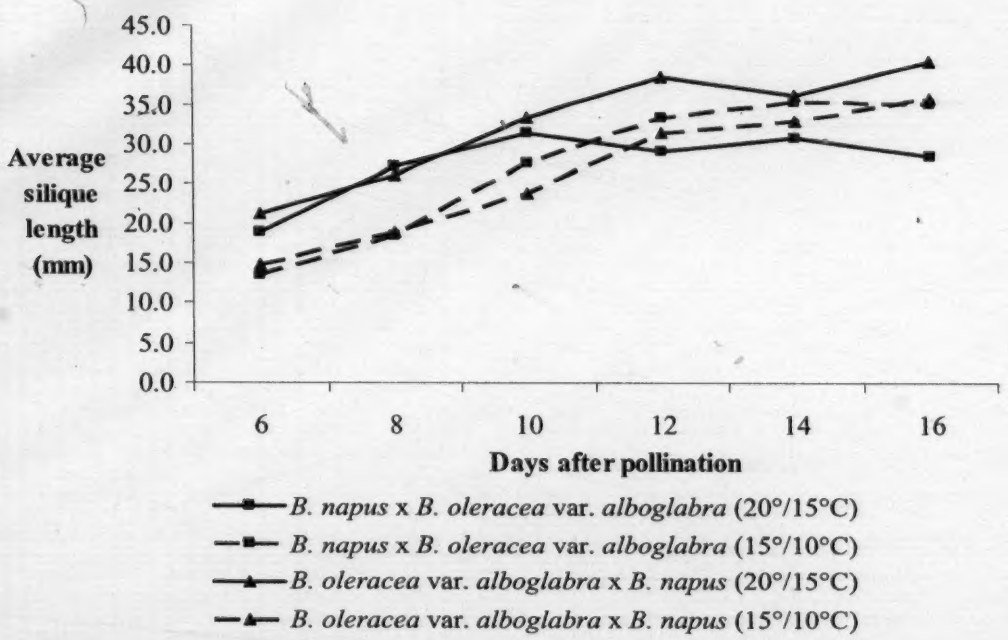

Figure 2-1. Average length of siliques of *B. napus* x *B. oleracea* var. *alboglabra* reciprocal interspecific crosses under two temperature conditions and harvested at six different ages.

2.3.1.2 Fertilized ovules

Self-pollination of *B. napus* yielded 17.0 (± 4.7) fertilized ovules per silique, while self-pollination of *B. oleracea* yielded 9.5 (± 5.8) fertilized ovules per silique (Table 2-1, Figure 2-2). In general, interspecific crosses using *B. napus* as the female parent yielded a lower number of fertilized ovules per silique (3.8 ± 2.3) than crosses using *B. oleracea* as the female (7.4 ± 3.8). No significant difference in the number of fertilized ovules was found due to growth condition (15°C vs. 20°C) when *B. napus* was used as the female parent in both crosses (Table 2-1). However, a significantly greater number of fertilized ovules per silique was obtained at the lower temperature when *B. oleracea* was used as the female parent (4.4 ± 1.9 vs. 9.8 ± 3.3, $t=4.76$, $P<0.01$, Figure 2-2). This difference was also evident for self-pollinated *B. oleracea*.

Table 2-1. Number of fertilized ovules for siliques harvested from 6 to 16 DAP following reciprocal interspecific crosses between *Brassica napus* and *B. oleracea* var. *alboglabra*

Cross	Temp. (°C)	No. pollinations	No. fertilized ovules	No. fertilized ovules / poll. (Mean ± SD)
Hi-Q	20/15	91	304	3.3 ± 3.2
x *B. oleracea*	15/10	115	309	2.7 ± 1.4
A01-104NA	20/15	114	340	3.0 ± 1.5
x *B. oleracea*	15/10	116	715	6.2 ± 3.8
B. oleracea	20/15	88	330	3.8 ± 2.3*
x Hi-Q	15/10	108	1002	9.3 ± 4.4
B. oleracea	20/15	92	454	4.9 ± 2.2*
x A01-104NA	15/10	123	1251	10.2 ± 3.2
Selfed	20/15	29	522	18.0 ± 3.1
B. napus	15/10	34	550	16.2 ± 5.9
Selfed	20/15	27	172	6.1 ± 3.4*
B. oleracea	15/10	30	388	12.9 ± 5.5

*P<0.05 for 20°/15°C vs. 15°/10°C (paired t-test).

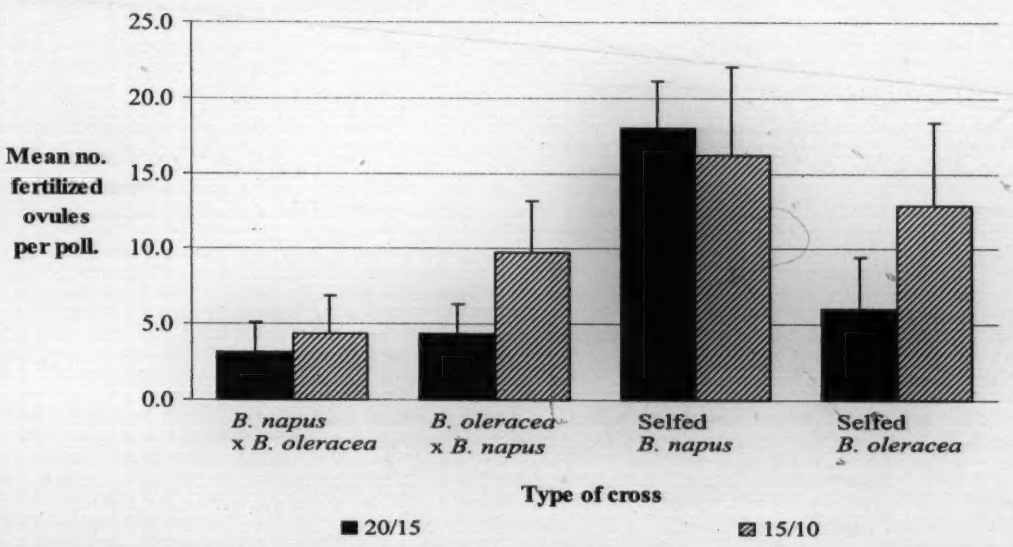

Figure 2-2. Number of fertilized ovules per pollination following reciprocal interspecific crosses between *Brassica napus* and *B. oleracea* var. *alboglabra* and under two temperature conditions. Average values of Hi-Q x *B. alboglabra* and A01-104NA x *B. alboglabra* (*B. napus* x *B. oleracea*); *B. alboglabra* x Hi-Q and *B. alboglabra* x A01-104NA (*B. oleracea* x *B. napus*) are given.

2.3.1.3 F_1 hybrid embryo production

Hybrid embryo yield of these interspecific crosses, viz. *B. napus* (Hi-Q) x *B. alboglabra*, *B. alboglabra* x Hi-Q, *B. napus* (A01-104NA) x *B. alboglabra*, and *B. alboglabra* x A01-104NA was very poor which restricted the use of individual cross data in statistical analysis. Therefore, data for the two crosses of *B. napus* x *B. oleracea* and *B. oleracea* x *B. napus* were pooled and subjected to statistical analysis.

Embryo yield was extremely low or almost zero at 6 to 8 DAP in all crosses. Using *B. napus* as the female parent, the greatest efficiency of embryo rescue occurred when siliques were harvested at 14 DAP and developed under 15°/10°C temperature, where 0.15 embryos per pollination were rescued (Figure 2-3). The greatest efficiency under 20°/15°C was at 10 DAP, where 0.11 embryos per pollination were rescued (Figure 2-3). On the other hand, using *B. oleracea* as female, the greatest number of embryos per pollination was obtained at 16 DAP under 20°/15°C, yielding 0.31 rescuable embryos per pollination (Figure 2-3). Furthermore, most (97%) embryos were obtained at 12 to 16 DAP for this cross. In general, the yield of rescuable embryos was significantly greater at higher temperature than at lower temperature (χ^2=15.94, P<0.01, Table 2-2) – despite a greater number of fertilized ovules obtained in siliques from lower temperature (Table 2-1). Only 0.7% of the fertilized ovules from lower temperature yielded rescuable embryos, while it was 5.2% of the ovules from higher temperature. Ovule culture greatly enhanced the efficiency of interspecific hybrid production, while no hybrid plants could be obtained *in vivo* from 288 pollinations (Table 2-3).

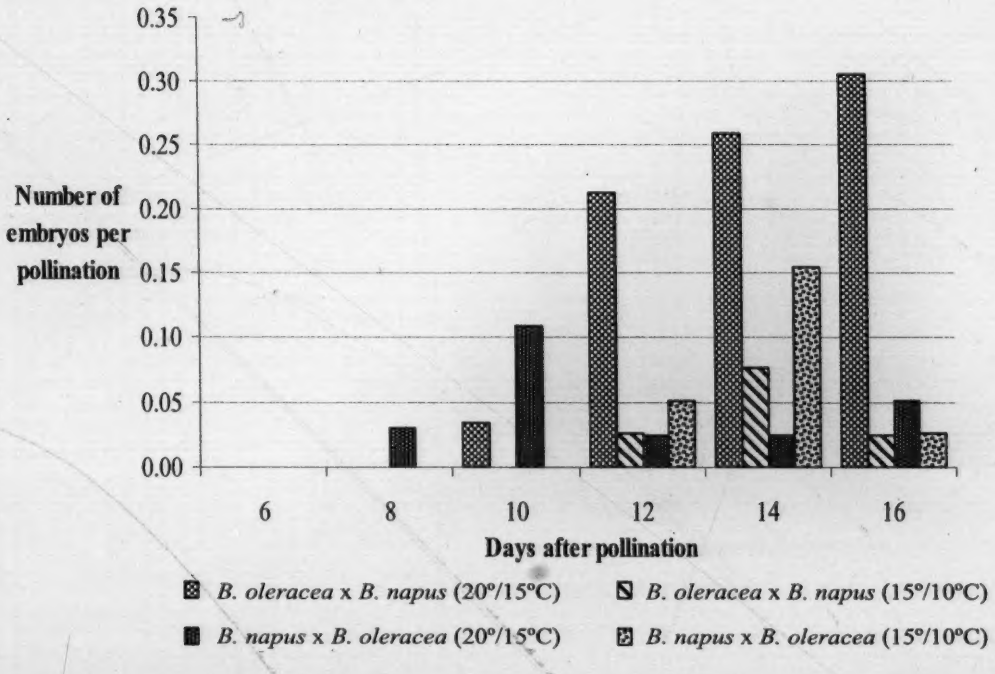

Figure 2-3. Hybrid embryo yield for the reciprocal crosses of *B. napus* and *B. oleracea* var. *alboglabra* from two temperature conditions and siliques harvested at six different dates. Average values of Hi-Q and A01-104NA presented as *B. napus*.

Table 2-2. Total number of hybrid embryos from siliques harvested during the whole period of 6 to 16 DAP and developed under two temperature conditions for reciprocal crosses between *B. napus* and *B. oleracea* var. *alboglabra*

Cross	*B. napus* x *B. oleracea* var. *alboglabra*		*B. oleracea* var. *alboglabra* x *B. napus*	
Temp. (°C)	20/15	15/10	20/15	15/10
No. pollinations	205	231	180	231
No. embryos produced	9	9	27	5
No. embryos / pollination	0.044	0.039	0.150^{y}	0.022^{z}

y vs. z = significantly different, $\chi^2 = 15.94$, $P<0.01$.

Table 2-3. *In vivo* interspecific hybrid production for reciprocal crosses between *B. napus* x *B. oleracea* var. *alboglabra*

Cross	No. pollinations	No. true hybrids
A01-104NA x *B. oleracea*	72	0
Hi-Q x *B. oleracea*	72	0
B. oleracea x A01-104NA	72	0
B. oleracea x Hi-Q	72	0

2.3.1.4 Confirmation of the F_1 hybrids

Nineteen C-genome specific primers, distributed throughout each of the nine linkage groups, were used (Appendix A). The marker sS2129, which showed good polymorphism, was used to confirm hybridity of the F_1 plants. This marker is located on the *B. napus* linkage group 15 (C-genome), and generates approximately 168 to 198 base pair fragments. A total of 62 embryos were obtained from reciprocal interspecific crosses. Of these, 46 (74.2%; Table 2-4) grew to maturity. All plants were tested by SSR markers and 34 were confirmed to be true hybrids, eight of which were from *B. napus* x *B. oleracea* crosses and the other 26 were from *B. oleracea* x *B. napus* crosses (Table 2-4, Figure 2-4).

Table 2-4. Number of F_1 hybrid plants obtained from rescued embryos

Cross	No. embryos	Plants grown to maturity	Confirmed hybrids
A01-104NA x *B. oleracea*	13	8	5
Hi-Q x *B. oleracea*	15	10	3
B. oleracea x A01-104NA	18	17	15
B. oleracea x Hi-Q	16	11	11
Total	62	46	34

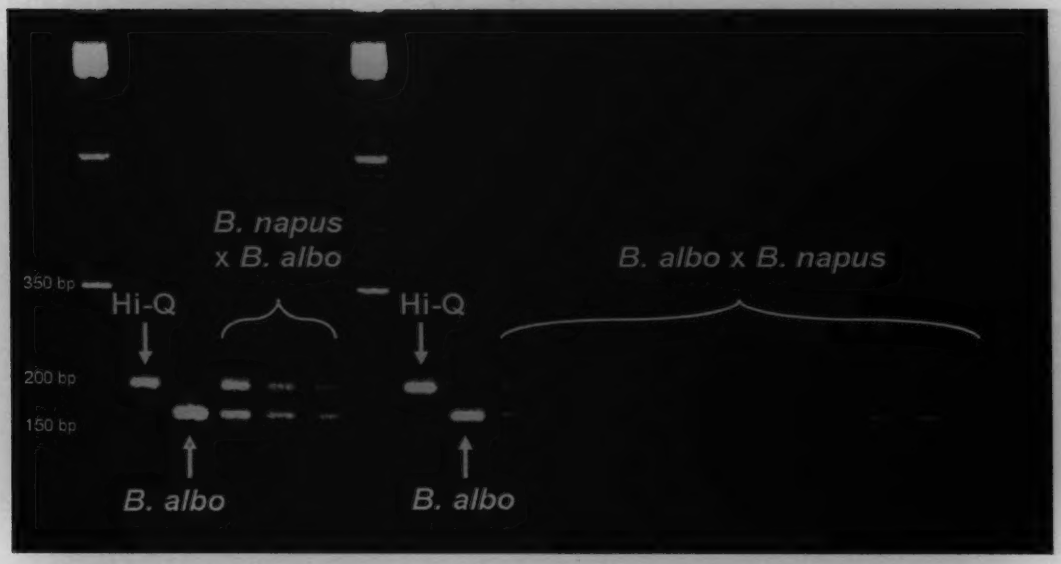

Figure 2-4. Microsatellite (SSR) marker amplification patterns of the parental species and their interspecific F_1 hybrids of Hi-Q x *B. oleracea* and *B. oleracea* x Hi-Q crosses. Parents are shown with arrows: Hi-Q (left), *B. alboglabra* (right).

2.3.1.5 F_1 plant morphology

Several key morphological traits distinguished *B. oleracea* var. *alboglabra* from *B. napus* lines 'Hi-Q' and 'A01-104NA' (Table 2-5). *B. alboglabra* is white-flowered, while *B. napus* is yellow-flowered. The flower buds of *B. alboglabra* are much larger in size than those of *B. napus*. The main stem of *B. alboglabra* is thicker in the middle in contrast to a thin and relatively uniform stem of *B. napus*. The leaves of *B. alboglabra* are larger, non-clasping, and leaf margin is almost smooth (undulated); while the leaves of *B. napus* are half-clasping with more pronounced dentation on the margins (Figure 2-5).

The flower colour of the F_1 hybrid plants was not as white as *B. alboglabra*, but slightly creamy white. The size of the flowers and buds tended to be intermediate of the parents. A chimeric flower, with three yellow petals and one white petal, was found on a *B. oleracea* x Hi-Q hybrid plant. Narrow stems and dented leaf margins, the characteristic of *B. napus*, was commonly observed in the reciprocal hybrids (Figure 2-5). The majority of hybrid plants tended to have leaf morphology intermediate of the parents. In general, the interspecific F_1 hybrid plants were significantly taller and flowered significantly later than average flowering date of the two parents (mid-parent) (Table 2-6). The F_1 hybrid plants produced very small quantity of seeds (0.01 to 0.04 g, Table 2-6) compared to their parents, although they generally produced greater numbers of siliques. This could be due to aneuploid nature of the hybrid plants.

Table 2-5. Flower colour and leaf morphology of the F_1 hybrid plants derived from reciprocal interspecific crosses between *B. napus* and *B. oleracea* var. *alboglabra*

		Flower colour		Leaf morphology[z]		
Cross	No. Plants	Yellow	White	N	I	A
A01-104NA x *B. oleracea*	5	0	5	1	2	2
Hi-Q x *B. oleracea*	3	0	3	2	1	0
B. oleracea x A01-104NA	15	0	15	1	10	4
B. oleracea x Hi-Q	11	0	11	1	8	2

[z]N= *B. napus* type – Half-clasping, lobed, dentation on margin.
A= *B. oleracea* var. *alboglabra* type – Non-clasping, absence of lobes, undulated margin.
I= Intermediate type – Has distinguishing characteristics of both parents.

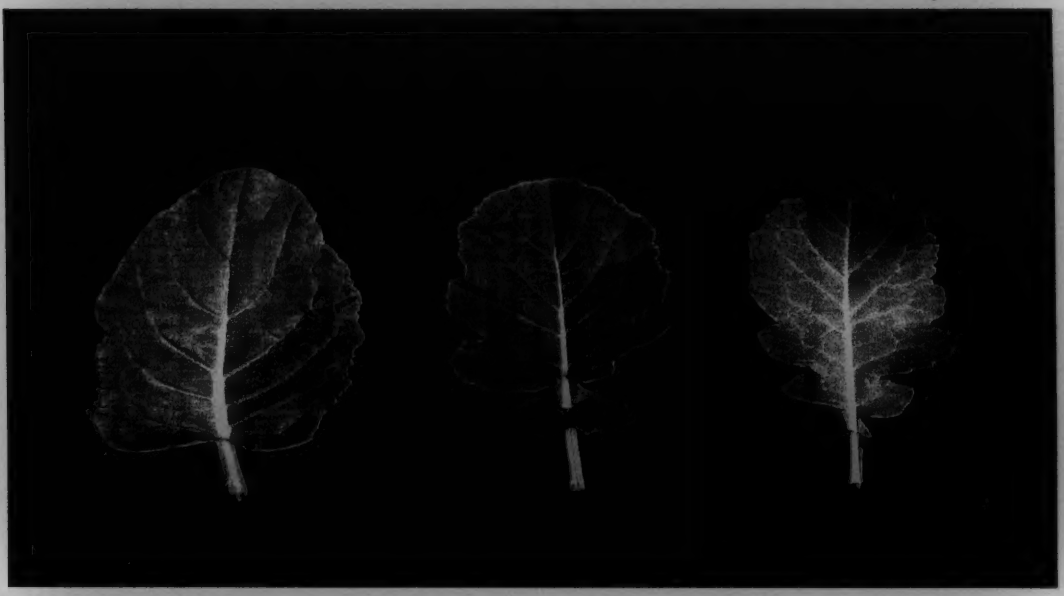

Figure 2-5. Leaf morphology of *B. oleracea* var. *alboglabra* (left), *B. napus* (A01-104NA) (right), and their interspecific F_1 hybrid plant (center).

Table 2-6. Agronomic traits of the F_1 hybrid plants (mean ± SD) derived from reciprocal interspecific crosses between *B. napus* and *B. oleracea* var. *alboglabra*

Parentage	No. plants	Days to flower[z]	No. siliques on main raceme	Plant height (cm)	Seed yield (g)
A01-104NA	3	41.7 ± 4.0	35.7 ± 9.5	106.3 ± 11.6	2.60 ± 1.25
Hi-Q	3	42.3 ± 3.1	27.7 ± 15.0	114.0 ± 4.4	3.55 ± 1.85
B. oleracea	3	63.0 ± 3.6	28.3 ± 11.0	96.7 ± 13.3	2.32 ± 0.49
A01-104NA x *B. oleracea*	5	68.8 ± 9.9*	45.2 ± 17.5	184.4 ± 21.1**	0.03 ± 0.05**
B. oleracea x A01-104NA	15	71.3 ± 17.6**	53.0 ± 24.7**	183.4 ± 26.5**	0.04 ± 0.04**
Hi-Q x *B. oleracea*	3	77.0 ± 25.7	37.0 ± 28.0	162.0 ± 49.1	0.01 ± 0.02**
B. oleracea x Hi-Q	11	65.4 ± 6.6**	47.6 ± 28.3*	182.9 ± 37.4**	0.03 ± 0.05**

[z]Days to flowering is defined as the number of days from plantlet transfer to soil-free growth medium to the opening of the first flower.
Significantly different at $^{*}P<0.05$, $^{**}P<0.01$ compared with the mid-parent value.

2.3.2 Backcrosses

Backcrossing was done on 31 F_1 plants using *B. oleracea* var. *alboglabra* as the male parent. Of these, 12 plants were grown in a greenhouse at 20-25°/18-20°C day/night temperatures and 19 plants were kept in a growth chamber set at 15°/10°C with 16 h photoperiod. Backcrossing was done on F_1 plants from all cross combinations and grown in both places. From 358 pollinations done on the F_1 plants in a greenhouse, only a single ovule was obtained, but no embryos could be rescued. However, significantly greater number of backcrossed ovules was obtained from the F_1 plants grown in a growth chamber. The F_1 plants from all crosses produced a similar number of fertilized ovules per pollination (0.76 to 0.80, Table 2-7), except *B. alboglabra* x Hi-Q where 0.45 ovules per pollination were obtained. Backcrosses performed on A01-104NA x *B. alboglabra* yielded the greatest number of embryos per pollination (0.107, Table 2-8). In general, the F_1 hybrid plants from the reciprocal crosses of *B. oleracea* x A01-104NA yielded a greater number of embryos than the F_1 plants from the *B. oleracea* x Hi-Q cross (Table 2-8). The *in vitro* ovule culture technique increased the rate of hybrid embryo production approximately 10-fold compared to *in vivo* seed set for backcrosses of *B. napus* x *B. oleracea* hybrid plants (Table 2-8).

Table 2-7. Number of fertilized ovules per silique from F_1 hybrid plants pollinated with *B. oleracea* var. *alboglabra* in a growth chamber

Cross	No. silique	No. ovules	No. ovules per silique
(A01-104NA x *B. alboglabra*) x *B. alboglabra*	87	68	0.78
(Hi-Q x *B. alboglabra*) x *B. alboglabra*	173	139	0.80
(*B. alboglabra* x A01-104NA) x *B. alboglabra*	240	182	0.76
(*B. alboglabra* x Hi-Q) x *B. alboglabra*	198	90	0.45
Total	698	479	0.69

Table 2-8. Efficiency of *in vitro* ovule culture technique vs. *in vivo* seed set for the production of interspecific BC_1 hybrids in a growth chamber

	In vitro plants			*In vivo* seed set		
Parentage[z]	No. crosses	No. embryos obtained	No. embryo per poll.	No. crosses	No. seeds	No. seeds per poll.
668	281	30	0.107	2213	19	0.009
669	379	29	0.077	1803	13	0.007
774	240	11	0.046			
867	198	4	0.020			
Total	1098	74	0.067	4016	32	0.008

[z]'668' - (A01-104NA x *B. alboglabra*) x *B. alboglabra*
'669' - (Hi-Q x *B. alboglabra*) x *B. alboglabra*
'774' - (*B. alboglabra* x A01-104NA) x *B. alboglabra*
'867' - (*B. alboglabra* x Hi-Q) x *B. alboglabra*

2.4 Discussion

Several investigations have been done by researchers in the past to improve the interspecific hybridization efficiency in the family *Brassicaceae* (Takeshita et al. 1980, Bajaj et al. 1986, Ayotte et al. 1987, Inomata 1993, Rahman 2004, Zhang et al. 2004). These studies primarily focused on the method of embryo rescue, time of harvest of hybrid embryos (DAP), and type of culture media. To the best of our knowledge, no study so far has been done to elucidate the effect of growing temperature and age of siliques on the efficiency of embryo rescue for the production of *Brassica* interspecific hybrids. The data presented in this paper suggest that the efficiency of embryo rescue in *B. napus* x *B. oleracea* var. *alboglabra* interspecific crosses depends greatly on the interaction between maternal genotype and growth condition (temperature). The slower growing species *B. oleracea* var. *alboglabra* yielded the greatest number of hybrid embryos under higher temperature and at 16 DAP. On the other hand, the relatively rapid growing species *B. napus* yielded the greatest number of hybrid embryos under lower temperature and at 14 DAP. This interspecific cross seems to be quite difficult to achieve, as reported by several authors. For example, U (1935) obtained 0.0033 hybrids per pollination and Chiang et al. (1977) obtained 0.00049 hybrids per pollination from *B. napus* x *B. oleracea* interspecific crosses under *in vivo* condition. This was also apparent from our study where no *in vivo* hybrid was obtained from 288 cross-pollinations. Thus, application of the ovule culture technique greatly enhanced the rate of hybrid production compared to *in vivo* hybrid seed set; and results of the present study can be applied for efficient production of interspecific hybrids of *B. napus* x *B. oleracea* crosses.

Nishiyama et al. (1991) reported that tetraploid x diploid crosses in the family *Brassicaceae* are often more successful when the tetraploid species is used as the female parent. This has been confirmed in several studies (Quazi 1988, Ripley and Arnison 1990, Choudhary et al. 2000, Rahman 2004). However, in our study, a greater number of hybrid plants (26 of 34, i.e. 76%) were obtained using the diploid species *B. oleracea* as the female parent. Chen et al. (1988) made reciprocal interspecific crosses between *B. oleracea* var. *alboglabra* and *B. rapa* for the resynthesis of *B. napus*, and found a higher rate of success when *B. oleracea* was used as the female parent. They suggested that the larger size ovule of this species eases handling of the materials under *in vitro* culture, and this might have contributed to the higher efficiency while using *B. alboglabra* as the female parent. This might also be a reason for greater success using *B. alboglabra* as the female parent in our study. It is interesting to note that the cross-pollinated siliques of *B. napus* were slow in development and contained a significantly lower number of fertilized ovules compared to self-pollinated siliques. On the other hand, cross-pollinated siliques of *B. oleracea* were almost equally developed and yielded a similar number of fertilized ovules under both temperature conditions. In general, cross-pollinated ovules of *B. napus* degenerated faster than the *B. oleracea* ovules.

Several investigators (U 1935, Honma and Summers 1976, Chiang et al. 1977, Ayotte et al. 1988) reported that F_1 hybrid plants from *B. napus* x *B. oleracea* crosses are often matroclinous in the early vegetative stages, while intermediate morphology emerges as the plants mature. This was also found for most of the hybrid plants in our study. Young vegetative hybrid plants predominantly resembled the maternal parent up to the 4 to 5 leaf stage, after which morphological traits from the paternal species became

evident. Therefore, waiting until bolting stage would be more effective for evaluation of leaf morphological traits. Most adult hybrid plants had morphological traits intermediate of the parents. Compared to the parents, greater variation was observed in the hybrids for several morphological traits, including days to flower, plant height, and the number of siliques on the main raceme. An unbalance of genes regulating these traits (haploid A-genome vs. diploid C-genome) might have contributed to the large variation observed in this population.

Chen et al. (1988) reported that the white flower colour of *B. oleracea* var. *alboglabra* is partially epistatic over the yellow flower colour of *B. rapa* in interspecific hybrids of these species. All F_1 hybrid plants produced in the present study had white flower colour. This suggests that the white flower colour gene of the C-genome of *B. oleracea* var. *alboglabra* is dominant over the yellow flower colour gene of the C-genome as well as epistatic over the flower colour gene of the A-genome of *B. napus*.

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Chapter 3

The inheritance of erucic acid in a self-pollinated backcross generation (BC_1S_1) of interspecific crosses between *Brassica napus* and *B. oleracea* var. *alboglabra*

3.1 Introduction

Brassica napus (AACC, 2n=38) is an amphidiploid species, carrying the A- and C-genomes from its progenitor species, *B. rapa* (AA, 2n=20) and *B. oleracea* (CC, 2n=18). Each of these two genomes is known to possess one locus responsible for production of erucic acid (C22:1) in seed oil (Dorrell and Downey 1964, Chen and Heneen 1989).

Several alleles controlling the synthesis of erucic acid in seed oil have been reported in the literature, viz. *e* (0%), E^a (10%), E^b (12-15%), E^c (30%), and E^d (3.5%) (Getinet et al. 1997). These alleles act in an additive (Harvey and Downey 1964) or partly dominant (Rahman et al. 1994) manner depending on the strength of the allele. For example, the *B. rapa* lines 'yellow sarson' and 'brown sarson' used by Dorrell and Downey (1964) contained 59% erucic acid; and when reciprocally crossed with a zero-erucic *B. rapa* line, the erucic acid content in F_1 seeds was almost intermediate or slightly higher (33% erucic acid) than the mid-parental value. This indicates that 'yellow sarson' and 'brown sarson' contain E^c alleles that contribute 30% erucic acid in the seed. However, partial dominance of this allele was more clearly suggested by Rahman et al. (1994), where they reported 41% erucic acid in F_1 seeds from a cross between 0.04% and 54% erucic acid parents. The diploid C-genome species *B. oleracea* var. *alboglabra* has been characterized as having an average seed erucic acid content of 40 to 50% (Chen et al. 1988, Chen and Heneen 1989, Rahman 2002), which could be under the genetic control

of a different allele that has not yet been characterized. Jönsson (1977) drew a generalized conclusion on the strength of the erucic acid alleles at the intraspecific level in *B. napus* and *B. rapa* that for erucic acid content below 30% the alleles show an additive effect, while at higher concentration partial dominance is more prevalent. The zero-erucic acid phenotype occurs when the loci are homozygous for the zero-erucic alleles.

Studies on the inheritance of erucic acid in *Brassica* species have often relied on crosses between high and low erucic acid genotypes within the same species (Dorrell and Downey 1964, Harvey and Downey 1964, Kondra and Stefansson 1965, Krzymanski and Downey 1969, Rahman et al. 1994, Getinet et al. 1997). In these reports, erucic acid content was studied in the F_1 and in different segregating populations, such as F_2, F_3, backcross, testcross, and tested against a hypothesized genetic model. However, the effect of different genomes on the synthesis of erucic acid in interspecific hybrids in *Brassicaceae* needs to be better understood. Rahman (2002) investigated the effect of the zero-erucic acid allele of the A-genome (*B. rapa* ssp. *trilocularis* var. 'yellow sarson') when combined with the high erucic acid alleles of the BC-genome of *B. carinata* (41%). He found a significantly lower level of erucic acid in the seeds of trigenomic allohexaploid interspecific hybrids (AABBCC) compared to its *B. carinata* parent (mean 33.4% vs. 41.3%), but not in trigenomic haploids (ABC). This indicates that the strength of the alleles could be affected by the genomic composition of the plant.

Petal (flower) colour in the diploid species *B. rapa* and *B. oleracea* has been found to be under monogenic control (Chen and Jönsson 1987, Rahman 2001). Chen and Jönsson (1987) reported that white petal colour of the *Brassica* C-genome is dominant to

yellow petal colour, and interspecifically epistatic to the yellow petal colour gene of the A-genome. Rahman et al. (2001) studied the segregation of erucic acid and petal colour in four doubled haploid (DH) populations derived from crossing of two white-petalled, intermediate erucic acid (26-28%) synthetic *B. napus* lines (white petal colour derived from *B. oleracea* var. *alboglabra*) with two yellow-petalled zero-erucic *B. napus* cultivars, and reported that 25 of the 26 yellow-petalled DH lines were free of erucic acid, while 33 of the 35 white-petalled lines contained erucic acid. This indicates that the white petal colour and erucic acid loci on the C-genome of *B. oleracea* var. *alboglabra* are linked, and the distance between these two loci to be 4.9 cM.

The objective of this study was to investigate the inheritance of erucic acid in a BC_1S_1 population derived from self-pollinations of (*B. napus* x *B. oleracea* var. *alboglabra*) x *B. oleracea* var. *alboglabra* BC_1 hybrids. The inheritance of flower colour was studied in relation to the inheritance of erucic acid. The BC_1 plant morphology and fertility were also examined.

3.2 Materials and methods

3.2.1 Parent material

Two yellow-flowered, zero erucic acid *B. napus* (AACC, 2n=38) doubled haploid lines, 'Hi-Q' and 'A01-104NA', and a white-flowered, high erucic (40.1% erucic acid) *B. oleracea* var. *alboglabra* (CC, 2n=18) inbred (F_7) line were used.

3.2.2 Experimental procedure

Interspecific crosses were made between *B. napus* and *B. oleracea* var. *alboglabra* and F_1 hybrids were produced through application of ovule culture technique. The detail of this technique is described in section 2.2.3. The F_1 hybrid plants were cross-pollinated with *B. oleracea* var. *alboglabra*, and the ovule culture technique was applied to generate backcross (BC_1) hybrids. The BC_1 plants were evaluated for pollen fertility as well as morphological characteristics, e.g. leaf lobes, stem thickness and petal colour; and were self-pollinated manually as well as under bag isolation to generate BC_1S_1 seeds. The half-seed technique of gas chromatographic fatty acid analysis was applied to determine the content of erucic acid in BC_1S_1 as well as in parental seeds.

3.2.3 BC_1S_1 seed set: manual vs. bag isolation

Two techniques were applied for self-pollination of the BC_1 plants to obtain BC_1S_1 seeds: manual self-pollination or self-pollination by bag isolation. Manual self-pollination was done by pollinating individual flower buds/stigmas (ca. one day prior to anthesis) of the BC_1 plants using pollen from the same plant. Self-pollination by bag isolation was done by covering individual plants with transparent, micro-perforated plastic bags and shaking bags daily to promote pollen transfer. Seed set under each method was determined by dividing the number of seeds obtained by the total number of pollinated flowers.

3.2.4 Pollen fertility

One unopened bud (ca. one day prior to anthesis) from each of the 10 BC_1 plants was used to estimate pollen fertility. The anthers were removed and crushed on a glass

slide containing 1 to 2 drops of 3% acetocarmine (w/v). The debris was removed and the slide was covered with a cover slip. Counts of pollen grains were made under a microscope at 200x magnification from four non-overlapping fields. Pollen grains were classified as either (i) viable, characterized by well-formed pollen grains and stained red, or (ii) non-viable, characterized by unstained and shrunken appearance. Pollen fertility was expressed as the percentage of viable pollen to the total number of pollen grains counted.

3.2.5 Fatty acid analysis

Seeds were placed on 110 mm #2 Whatman filter paper in 100 x 15 mm sterile petri dishes under room temperature (22 to 25°C). Distilled water was used to saturate the filter paper and germinate the seeds. The majority of the seeds were germinated in 24 to 72 h. One cotyledon of each of the newly germinated seeds were dissected and transferred in a 10 x 75 mm glass test tube for fatty acid analysis. The remainder of the seed was transferred to a 100 x 15 mm petri dish containing autoclaved silica sand, and each dish was watered as needed. Petri dishes were placed in a growth room set at room temperature (22 to 25°C) with 12 h photoperiod and photon flux density of 30 μE (mV) m^{-2} s^{-1} until the embryos were fully germinated and roots were developed. Seedlings were transplanted to soil-free growth medium (Stringam 1971) in a growth chamber [15°/10°C day/night temperature, 16 h photoperiod, 450 μE (mV) m^{-2} s^{-1} photosynthetic flux density at plant level] and were covered with transparent plastic tubes for three to four days to harden and acclimatize them to the new environment.

For fatty acid analysis, the cotyledons were dried at 80°C using Thermolyne Dri-Bath for one hour. For fatty acid analysis of lipids by gas chromatography (GC), it is

necessary to convert the fatty acids to low molecular weight non-polar derivatives, such as methyl esters. Therefore, the cotyledons were immersed in 1.2 mL Na^+ methylating solution and 0.25 mL hexane solvent and crushed with a glass rod (for extraction of oil and conversion to methyl esters). The tubes were capped and placed in the dark for 30 min to allow a methylation reaction to take place. A 20% NaCl solution (1 to 2 mL) was added to maximize the recovery of the short chain fatty acids (i.e. C12, C14 and C16). The hexane solvent (containing methyl esters) was transferred to the gas chromatography vials containing 250 µL glass BMI/spring inserts, and was evaporated to approximately 100 µL to increase the concentration of fatty acid methyl esters for ease of fatty acid analysis/detection. Gas chromatography was performed using a Hewlett-Packard chromatograph (model 6890N) equipped with a flame ionization detector. A DB-WAX (crosslinked polyethyleneglycol) column was used to obtain greater peak resolution and adequate separation, ensuring measurement of each individual fatty acid.

3.2.6 Erucic acid segregation

The digenomic triploid genomic composition of the *B. napus* x *B. oleracea* F_1 hybrid plants (CCA) makes it difficult to predict any definite segregation pattern in the BC_1S_1 generation. In meiosis of the F_1 hybrid plants, the diploid set of the C-genome chromosomes should theoretically follow a normal segregation; while the haploid set of ten A-genome chromosomes would segregate randomly and be included in the gametes in variable numbers ranging from 0 to 10. Backcrossing of the F_1 plants with *B. oleracea* var. *alboglabra* (CC) could therefore result a BC_1 population composed of variable genotypes ranging from 2n (CC) to 2n + 1 to 10 A-genome chromosomes. Meiosis in BC_1 plants would result in gametes with variable number of A-genome chromosomes (0

to 10), and self-pollination of the BC_1 population would result BC_1S_1 seeds with genetic constitution ranging from 2n (CC) to 2n + 1 to 20 A-genome chromosomes.

The genomic composition of the F_1 plants in respect to erucic acid alleles would be $C^+C^0A^0$ (C^+ = high erucic acid allele from *B. oleracea*; C^0 and A^0 = zero-erucic acid alleles from *B. napus*). In a diploid C-genome genotype heterozygous for erucic acid alleles (C^+C^0), a normal disomic segregation would be expected to occur. A heterozygous F_1 plant (C^+C^0) backcrossed with a homozygous parent (C^+C^+) would be expected to produce C^+C^0 and C^+C^+ genotypes in a 1:1 ratio in the BC_1 population (Figure 3-1). Random self-pollination of the BC_1 plants would result BC_1S_1 seeds in a proportion of 5 C^+C^+: 2 C^+C^0: 1 C^0C^0 (Figure 3-1). However, in our BC_1S_1 population derived from digenomic triploid $C^+C^0A^0$, inclusion of the A-genome chromosome carrying the zero erucic acid allele would affect the content of erucic acid to be produced by the C-genome alleles (C^+C^+ and C^+C^0). This may further be affected by the doses of the A-genome allele, e.g. $C^+C^+A^0$ and $C^+C^+A^0A^0$ genotypes would be expected to have different contents of erucic acid.

Several seed genotypes would be possible in BC_1S_1: C^+C^+, C^+C^0, C^0C^0, $C^+C^+A^0$, $C^+C^0A^0$, $C^0C^0A^0$, $C^+C^+A^0A^0$, $C^+C^0A^0A^0$, and $C^0C^0A^0A^0$. Possible phenotypes of the seeds could be predicted based on the reports by earlier researchers. The genotypes C^0C^0, $C^0C^0A^0$, and $C^0C^0A^0A^0$, where no alleles capable of producing erucic acid are present, are expected to have a zero-erucic acid phenotype. The genotype C^+C^+ carrying only the erucic acid producing alleles is expected to have erucic acid content similar to that of the *B. alboglabra* parent (mean 40.1% ± 0.36). The zero-erucic allele of the A-genome (A^0) lowers the content of erucic acid in the interspecific hybrid seeds carrying the C-genome

erucic acid allele (C^+) (Rahman 2002). Based on this, it is presumed that the genotype $C^+C^+A^0$ would have a lower content of erucic acid than the *B. alboglabra* parent – a phenotype of 29.1 to 33.2% erucic acid is assumed (Table 3-1). Chen et al. (1988) crossed zero-erucic turnip rape (*B. rapa*) with *B. oleracea* var. *alboglabra*, and found that the resynthesized *B. napus* line (genome constitution $C^+C^+A^0A^0$) contained 20.0% to 28.3% erucic acid (mean 25.8%). Dorrell and Downey (1964) and Rahman et al. (1994) crossed zero and high erucic (50-60%) *B. rapa* lines and reported that erucic acid content in the hybrid seeds (A^+A^0 genotype) was equal to or slightly higher than the mid-parent value. Therefore, genotypes C^+C^0 and $C^+C^+A^0A^0$ would be expected to have erucic acid content of 20.0 to 28.9% (Table 3-1). The $C^+C^0A^0$ genotype is expected to be lower than the mid-parent (20.1%) – a phenotype of 16.0 to 19.3% erucic acid is assumed (Table 3-1). Chen et al. (1988) made crosses between a synthetic *B. napus* line (containing C-genome from *B. alboglabra*) and a zero-erucic *B. napus* breeding line, and reported 10.2 to 14.0% erucic acid in the resulting hybrid seeds (genome constitution $C^+C^0A^0A^0$). In the present study, seeds with 7.8 to 15.3% erucic acid are assumed to be of $C^+C^0A^0A^0$ genotype (Table 3-1).

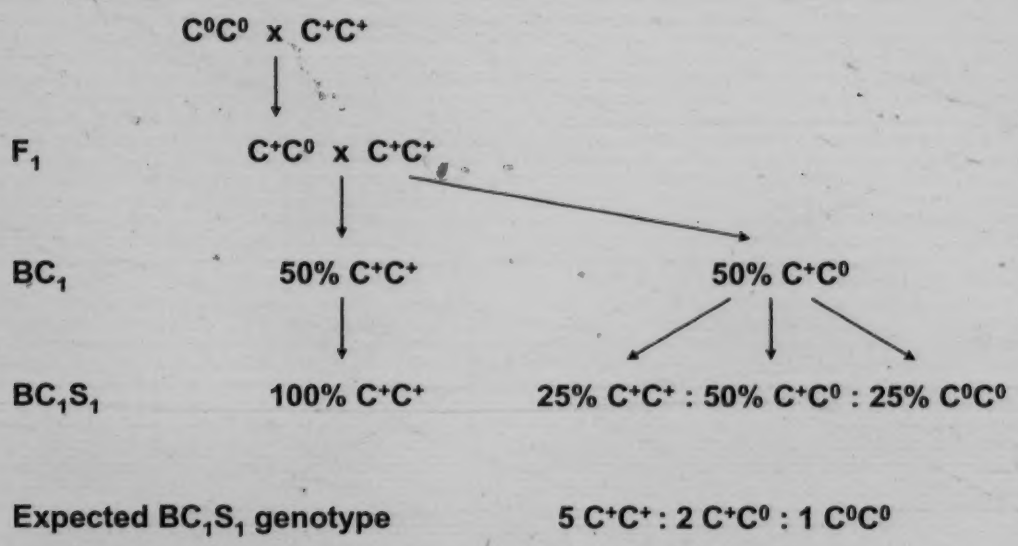

Figure 3-1. Expected genotypic frequencies in the BC_1S_1 generation in a diploid *B. oleracea* genotype segregating for C-genome erucic acid alleles.

Table 3-1. Expected erucic acid phenotypes of the BC_1S_1 seed genotypes with zero, one, or two A-genome chromosomes carrying the zero erucic acid allele (A^0)

Possible BC_1S_1 genotypes[z]	Expected phenotypes[y]
C^0C^0, $C^0C^0A^0$, $C^0C^0A^0A^0$	0.1-2.7
$C^+C^0A^0A^0$	7.8-15.3
$C^+C^0A^0$	16.0-19.3
C^+C^0, $C^+C^+A^0A^0$	20.0-28.9
$C^+C^+A^0$	29.1-33.2
C^+C^+	36.7-40.0

[z] C^0 = zero-erucic allele from *B. napus* parent; C^+ = erucic acid allele from *B. alboglabra* parent; A^0 = zero-erucic allele from A-genome of *B. napus* parent.
[y] BC_1S_1 seed erucic acid content (%).

3.2.7 Statistical analysis

A chi-square test for specified proportions using proc FREQ in the SAS system (Statistical Analysis System, Inc. 1999) was used to test normal disomic vs. observed segregation in BC_1S_1 seeds for erucic acid content.

3.3 Results

3.3.1 BC_1 morphology

Thirty-eight BC_1 plants were evaluated for morphological characteristics and all were white flowered (Table 3-2). Seventeen plants resembled the *B. oleracea* parent, while the remaining plants were either intermediate or *B. napus*-type (Table 3-2). Phenotypically 28 (74%) of the 38 plants were considered to be sterile (zero to extremely low production of viable pollen). Pollen fertility in the 10 BC_1 plants that produced visible pollen ranged from 11.7 to 90.8% (Table 3-3). Seed set on self-pollination was examined in nine of the 10 fertile plants. The efficiency of manual self-pollination was found to be 10-fold greater than self-pollination by bag isolation for the production of BC_1S_1 seeds (0.205 vs. 0.019, Table 3-4).

Table 3-2. Flower colour and plant type of the (*B. napus* x *B. alboglabra*) x *B. alboglabra* BC_1 hybrid plants

		Petal colour[z]		Plant type[y]		
Parentage	No. plants	W	Y	A	I	N
(A01-104NA x *B. alboglabra)* x *B. alboglabra*	24	24	0	10	7	7
(Hi-Q x *B. alboglabra)* x *B. alboglabra*	14	14	0	7	4	3
Total	38	38	0	17	11	10

[z]W = white (*B. alboglabra* type); Y = yellow (*B. napus* type).
[y]Based primarily on leaf and stem morphology, as per the following criteria:
N = *B. napus* type – half-clasping leaves, lobed, dentation on the margin, stems narrow and uniform; A = *B. alboglabra* type – non-clasping leaves, absence of lobes, undulated margin, stems thick in the middle; I = intermediate type – has distinguishing characteristics of both parents.

Table 3-3. Pollen fertility in 10 BC_1 plants of (*B. napus* x *B. alboglabra*) x *B. alboglabra*

Parentage	Plant no.	Viable pollen (%)
(A01-104NA x *B. alboglabra*) x *B. alboglabra*	.001 p5	46.3 ± 3.7
(A01-104NA x *B. alboglabra*) x *B. alboglabra*	.001 p6	38.7 ± 8.0
(A01-104NA x *B. alboglabra*) x *B. alboglabra*	.001 p8	47.3 ± 4.1
(A01-104NA x *B. alboglabra*) x *B. alboglabra*	.002 p3	11.7 ± 5.5
(A01-104NA x *B. alboglabra*) x *B. alboglabra*	.002 p4	28.6 ± 3.6
(A01-104NA x *B. alboglabra*) x *B. alboglabra*	.002 p8	46.3 ± 4.2
(A01-104NA x *B. alboglabra*) x *B. alboglabra*	.002 p9	62.2 ± 3.2
(A01-104NA x *B. alboglabra*) x *B. alboglabra*	.002 p12	90.8 ± 1.8
(Hi-Q x *B. alboglabra*) x *B. alboglabra*	.002 p6	38.8 ± 2.9
(Hi-Q x *B. alboglabra*) x *B. alboglabra*	.003 p6	43.8 ± 2.3

Table 3-4. Seed set in BC_1 plants (=BC_1S_1 seeds) from manual self-pollination and self-pollination by bag isolation

		Manual pollination			Bag isolation	
Parentage[z]	No. plants	No. poll.	No. seeds	No. seeds / poll.	No. flower isolated	No. seeds / flower
'668'	7	521	100	0.192	2777	0.021
'669'	2	84	24	0.286	952	0.013
Total	9	605	124	0.205	3729	0.019

[z]'668' = (A01-104NA x *B. alboglabra*) x *B. alboglabra*.
'669' = (Hi-Q x *B. alboglabra*) x *B. alboglabra*.

3.3.2 BC_1S_1 erucic acid inheritance

A class of zero-erucic acid (0 to 2% erucic acid) can easily be distinguished, where 19 of the 109 (17.4%) seeds fall into this class (Figure 3-2). Only three seeds had >30% erucic acid, i.e. 2.8% of the total number of seeds (Figure 3-2). In the remaining 87 (79.8% of total) seeds, the content of erucic acid ranged from 7 to 30% (Figure 3-2). The

segregation for erucic acid in BC_1S_1 seeds deviated from the normal disomic segregation that would be expected based on only the C-genome erucic acid alleles (χ^2=195.4, P<0.01, Figure 3-3). The observed number of seeds in the high erucic acid class (33 to 40%) was significantly lower while it was higher for the intermediate erucic acid class (7 to 30%) than the number of seeds that would be expected in these two classes based on segregation of only the C-genome erucic acid alleles (Figure 3-3). The zero-erucic acid class had also slightly greater number of seeds than expected (19 vs. 13.6, Figure 3-3). Within the intermediate class, the greatest number of seeds fell within the phenotypic classes presumed to be produced by genotypes with two A-genome erucic acid alleles: 36 ($C^+C^0A^0A^0$, 7.8 to 15.3% erucic acid) and 33 (C^+C^0 and $C^+C^+A^0A^0$, 20.0 to 28.9% erucic acid) seeds fell into these classes. On the other hand, a smaller number of seeds were found within the phenotypic classes expected to be produced by the genotypes having only one dose of the A-genome erucic acid allele: 11 ($C^+C^0A^0$, 16.0 to 19.3% erucic acid) and 7 ($C^+C^+A^0$, 29.1 to 33.2% erucic acid) seeds fell into these classes.

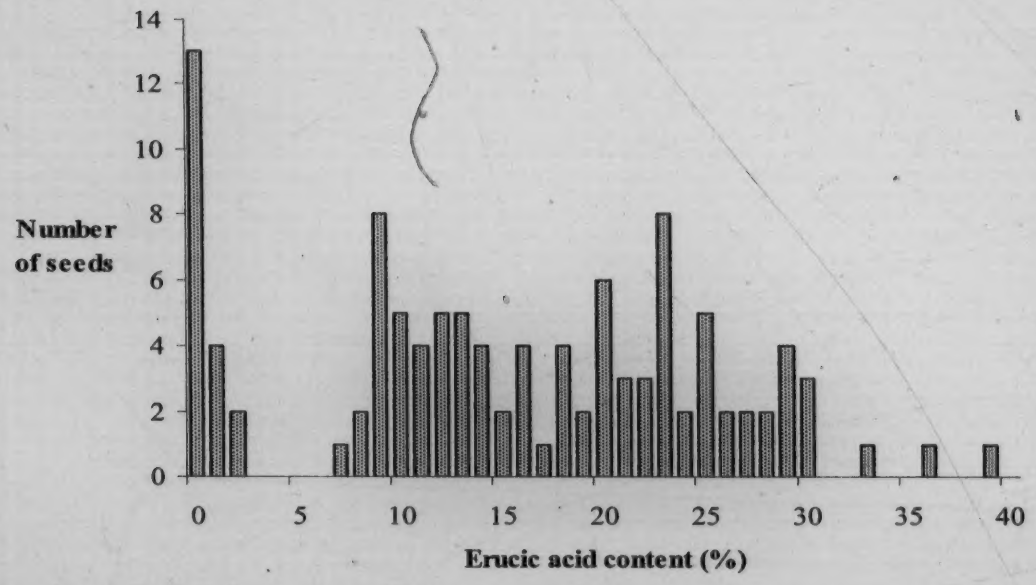

Figure 3-2. Distribution of the BC_1S_1 seeds for erucic acid content (n=109).

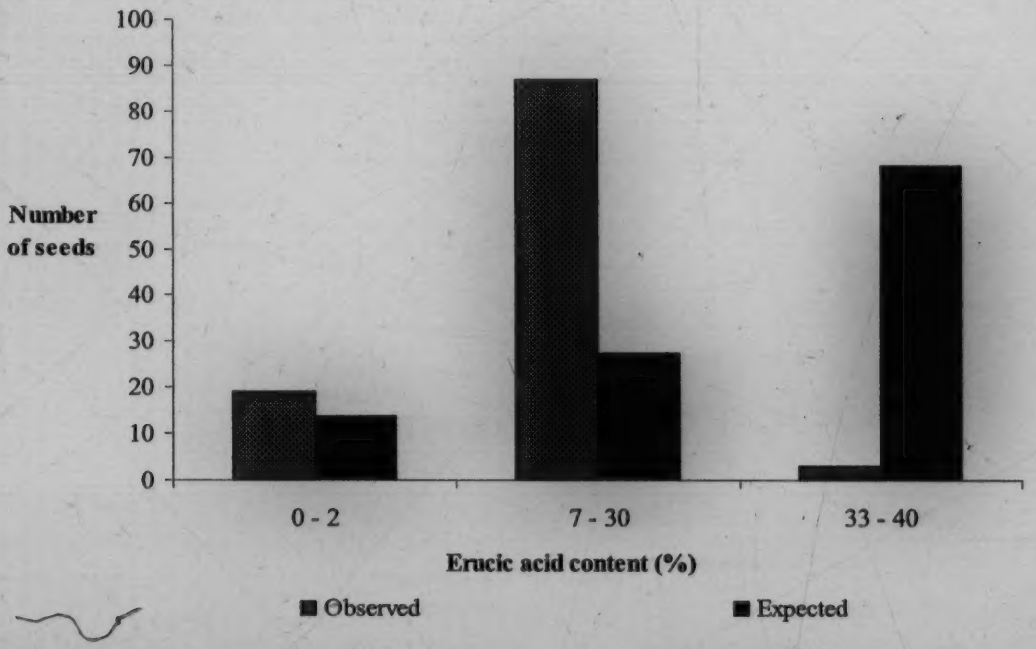

Figure 3-3. Observed number of BC_1S_1 seeds for three different erucic acid classes compared to the number of seeds to be expected from normal disomic segregation based on only the C-genome erucic acid alleles. BC_1S_1 seeds derived from self-pollination of (*B. napus* x *B. oleracea* var. *alboglabra*) x *B. oleracea* var. *alboglabra* hybrid plants.

3.3.2.1 Flower colour and erucic acid

Ninety-two BC_1S_1 plants were examined for petal colour of which 77 had white petal and 15 had yellow petal. This is in agreement with the expected 7:1 segregation for white:yellow petal colour (χ^2=1.22, P>0.05). Of the 109 BC_1S_1 seeds analyzed for erucic acid, 90 seeds contained this fatty acid (range 7.85-39.86, mean 19.09) and the remaining 19 were almost free from this fatty acid (range 0.11-2.71, mean 0.81), which is also in agreement with the expected 7:1 segregation (χ^2=2.42, P>0.05). Of 15 yellow-petalled BC_1S_1 plants, 12 (80%) were zero/low erucic; while of the 77 white-petalled plants, 73 (95%) contained erucic acid (Table 3-5). Thus, recombinant types were 7.6% of the total population.

Table 3-5. Segregation for petal colour and erucic acid content in BC_1S_1 population derived from self pollinations of (*B. napus* x *B. oleracea* var. *alboglabra*) x *B. oleracea* var. *alboglabra* BC_1 plants

	Yellow petal		White petal		Total
	-	+	-	+	
No. plants	12	3	4	73	92

'-' denotes absence of erucic acid (<3%); '+' presence of erucic acid (>7%).

3.4 Discussion

Pollen fertility in the BC_1 plants was generally low. Some of the plants produced almost no visible pollen. Pollen fertility in the 10 pollen-producing BC_1 plants ranged from 11.7 to 90.8% with a mean of 45.5%. Most of the BC_1 plants theoretically would be aneuploid and produced gametes with variable numbers of chromosomes. This would have resulted in low levels of pollen fertility in the BC_1 plants. Ayotte et al. (1988) also reported low pollen fertility in (*B. napus* x *B. oleracea*) x *B. oleracea* BC_1 hybrids (mean

24.1%, range 0 to 91.5%). In the present experiment, self-pollination by bag isolation resulted in extremely low numbers of seeds per pollination (0.019, Table 3-4). However, manual self-pollination increased the efficiency of BC_1S_1 seed production by 10-fold (Table 3-4). Thus, our results suggest that in interspecific hybrids, where pollen viability is low, significantly higher number of seeds can be obtained when flowers are manually self-pollinated.

Considering normal disomic segregation with only the C-genome erucic acid alleles, a genotypic ratio of 5 C^+C^+: 2 C^+C^0: 1 C^0C^0 would be expected in the BC_1S_1 generation (Figure 3-1). The C^+C^+ genotype would be expected to give a phenotype similar to the *B. oleracea* var. *alboglabra* parent (40.1% erucic acid); while the C^0C^0 genotype will result zero-erucic acid phenotype. The C^+C^0 genotype is expected to give a phenotype equal to or slightly higher than the mid-parent value (20.0 to 28.9% erucic acid, Table 3-1). However, in the *B. napus* x *B. oleracea* F_1 as well as in the (*B. napus* x *B. oleracea*) x *B. oleracea* BC_1 hybrids, aneuploid gametes carrying variable numbers of the A-genome chromosomes are expected to be produced. Therefore, in addition to segregation of the two C-genome erucic acid alleles, segregation for the A-genome zero-erucic allele would be expected, where zero to two recessive alleles can be inherited in the BC_1S_1 seeds. The presence of one or two recessive (A^0) alleles would lower the overall erucic acid content in the BC_1S_1 seeds. This has been, in fact, observed in the BC_1S_1 seeds. The observed number of seeds in the high erucic acid class was significantly lower than the expected number; while this was significantly higher in the intermediate class than the number that would be expected based on segregation of only the C-genome erucic acid alleles (Figure 3-3). This clearly suggests that a large

proportion of BC_1S_1 seeds with a C^+C^+ genotype must have inherited either one or two recessive A-genome erucic acid alleles. Furthermore, within the intermediate erucic acid class, the greatest number of seeds fell within phenotypic classes expected to be made up of genotypes with two recessive A-genome alleles. This indicates that the aneuploid gametes produced by the F_1 and BC_1 plants and carrying greater numbers of A-genome chromosomes may have been more viable than those with a lower number of A-genome chromosomes. This was also evident from the occurrence of a greater number of *B. napus* and intermediate-type plants in the BC_1S_1 generation. Fernandez-Escobar et al. (1988) also reported that aneuploid gametes containing higher numbers of chromosomes were more viable in the F_2 and BC_1 generations of interspecific crosses between *B. napus* (AACC, 2n=38) and *B. carinata* (BBCC, 2n=34). In our study, only one seed approached the phenotype of the *B. alboglabra* parent for erucic acid content (39.9% erucic acid). Chen and Heneen (1989) found that in the F_2 generation of a cross between zero- and high (56.5%) erucic acid *B. rapa* cultivars, no seeds with a level of erucic acid similar to the high parent could be recovered. They hypothesized that the high erucic alleles may function more effectively in the genetic background of its parent rather than the cultivar with which it was crossed. This, as well as possible aneuploid makeup of the seeds ($+A^0$, $+A^0A^0$), might be the reason for the extremely low number of seeds that approached the erucic acid levels of the *B. oleracea* parent in our study.

All BC_1 plants had white petal colour, confirming the dominant nature of the white petal colour gene in the *B. oleracea* var. *alboglabra* C-genome, as described by Chen and Jönsson (1987), and evidenced in the F_1 plants (section 2.3.1.5). The segregation for erucic acid and petal colour in the BC_1S_1 generation indicated that the high erucic acid

gene and the white petal colour gene of the C-genome of *B. oleracea* var. *alboglabra* are linked at a distance of 7.6 cM. This is in general agreement with Rahman et al. (2001), who found 4.9% recombination between these two gene loci. The results of this study can be applied in selecting zero-erucic *B. oleracea*-type plants from a segregating population where the yellow petal colour can provide a convenient morphological marker for such selection.

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Chapter 4

Conclusions

4.1 Conclusions

The primary contributions arising from these studies can be summarized as follows:

- Silique age (DAP) and growth condition (temperature) affected the efficiency of interspecific hybrid production through application of *in vitro* ovule culture technique in reciprocal *B. napus* x *B. oleracea* var. *alboglabra* interspecific crosses.

- Using *B. napus* as the female parent, siliques harvested at 14 DAP under 15°/10°C yielded the greatest number of rescuable embryos per pollination (0.15); while under higher temperature (20°/15°C) greatest efficiency was achieved at 10 DAP, where 0.11 embryos per pollination was obtained.

- Using *B. oleracea* var. *alboglabra* as the female, the greatest efficiency was achieved under higher temperature (20°/15°C) with siliques at the age of 16 DAP, yielding 0.31 embryos per pollination.

- Application of the *in vitro* ovule culture technique greatly increased the efficiency of production of interspecific F_1 and BC_1 hybrid plants compared to *in vivo* seed set.

- For generation of BC_1S_1 seeds, manual self-pollination of BC_1 plants increased the rate of seed production by 10-fold compared to self-pollination under bag isolation.

- Gametes produced in F_1 and BC_1 plants carrying greater numbers of the A-genome chromosomes appeared to be more viable than those with a lower number of A-genome chromosomes, as evidenced from the segregation of erucic acid alleles and morphology of the plants in BC_1 generation population.

- The zero-erucic acid phenotype was recovered in 17% of the BC_1S_1 seeds.

- The white petal colour gene of the C-genome of *B. oleracea* var. *alboglabra* was confirmed to be dominant over the yellow petal colour gene of *B. napus* and linked with the high erucic acid allele at a distance of ca. 5-7 cM.

The knowledge gained from these studies can be applied by other researchers to increase the efficiency of hybrid production of *B. napus* x *B. oleracea* var. *alboglabra* interspecific crosses. These studies also allowed us to better understand the inheritance pattern of erucic acid in an interspecific hybrid population (BC_1S_1), and in particular the rate at which zero-erucic phenotypes occur in this self-pollinated backcross generation. In addition, knowledge of the dominant nature of the petal colour gene and its linkage with the gene responsible for the synthesis of erucic acid in the C-genome of *B. alboglabra* can be used in initial selection of plants for zero-erucic acid by selecting yellow petal colour type. However, this needs to be confirmed by erucic acid analysis as a small amount of recombination between these two loci can occur.

Our current knowledge of the optimum growth condition and age of silique for efficient production of interspecific hybrids from *B. oleracea* var. *alboglabra* x *B. napus* cross can be further extended by studying silique ages beyond the 16 DAP and at temperatures higher than 20°/15°C.

Appendix A

Table A-1. Microsatellite primers used for evaluation of the C-genome of *B. napus* and *B. oleracea* var. *alboglabra* for polymorphism

Primer name	Linkage group	Expected Product Size	Forward Sequence (5' to 3')	Reverse Sequence (5' to 3')
sN9425	n11	362-365	CGC CGA CTT TAA AGA GAT GG	TCC TTG GGT TGG TCA CTG AT
sS1867A	n11	199-273	TCA CCA GGG CTG ATC TAT GA	GGG TGA TCT AGA TGG GTG AGT G
sN3514f	n12	105-122	CTA AGA AGG AAC CGG GAA GG	AAC CGG AAT CAG AAC ACA CA
sNRA56	n13	265	AAT CTT CGC ATA CCC ATT CG	GTT TGA CGC GGA TAA CCA TC
sN2032	n13	328-422	CAC CAT CAC CAC CTT CAC AA	TGG TTC ACT CAT GTC TCC GA
sS2277	n14	219-225	GAT CTG CGG TAG GAA TCG AA	CGT GCT ACA TAA TAG GGA AAA ACC
sN2552	n14	278-344	CCA TCA AAA CTC CTC TGG GA	TGG AGA TGC GAC TCA AAG TG
sORH13	n15	203-274	CCT GAT GTT TTG GTT GTG TCA	TCA CTG TGT TTA CTT GCG CC
sS2129	n15	168-198	GAG ACA TAG ATG AGT GAA TCT GGC	GTG GCC CAT TAG TTG TGG AC
sS2131	n15	189-200	TTG CTT CCA GAG ATC TGG TTC	TGC GAC TAC TAA ATT GTC GTG TT
sORH06	n16	419-437	CCT TAC TGG CTG CAG CTT TC	CGC TGG AAA CAC TTG TCA GA
sS2486	n16	338	AAA TGG GAA CGA GGG AAA GT	GCC TTT GGG TCA TCT GGA AT
sORD31	n16	190-192	TCG AAA GTG ATT AGG GTT TAT CG	CCC GAT CAG CTC AAC CTA AG
sN0706	n17	376-389	TCC GAC GGT CAA GAT TAA GG	GGC TGT GGT GGA TCT AGG AA
sNRH63	n17	113-129	GAA TCA GTC ACC AGG GGA GA	CCA TCG CGG TAA TTA AAC CT
sS2331B	n18	127-141	TGT CCT GTT TTC TGT GCT GG	GCC AAC GCT AGT TTT GCT TC
sORB29A	n18	100	CCT CTG TTT CCA TGG AGT GG	AAT GAT CAG GAG GAA CGT GG
sN2016	n19	264-271	GTG AGG AAA CGC AAC AGT GA	GGG TGG ATT GGA TGT ACC TG
sNRG42	n19	205-253	TCG TGG GGA TTA GTC TGA GC	ATC CCG AGT GAC AAA AAT TG